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Electron Microscopy of Chloramphenicol-treated *Escherichia coli*

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Thin sections of *Escherichia coli* were examined by electron microscopy at sequential intervals after addition and then removal of chloramphenicol. The first changes, occurring at 1 hr after exposure to the drug, were disappearance of the ribosomes and aggregation of the nuclear material toward the center of the bacteria. At 2 hr, aggregates of abnormal cytoplasmic granules first appeared and subsequently increased in size. By 23 hr, amorphous, electron-dense material had accumulated within, and at the periphery of, the nuclear matrix. With the removal of chloramphenicol, the bacteria became normal in appearance, passing through a series of stages that were sequential but not synchronous. At 145 min after removal of chloramphenicol, bacteria were encountered in the process of abnormal division. The influence of deoxyribonucleic acid and ribonucleic acid synthesis, and of energy metabolism, upon the changes seen electron microscopically in chloramphenicol-treated cells, was investigated by selectively inhibiting these functions with hydroxyurea, azauracil, and sodium azide, respectively.

The biochemical events which result from exposure of growing bacteria to chloramphenicol (CM) have been the subject of intensive investigation. CM is an antibacterial agent which primarily inhibits protein synthesis (10), apparently by interfering with the growth of nascent protein chains (15, 33). The synthesis of deoxyribonucleic acid (DNA) in CM-treated cells continues for some time at a reduced rate and then stops (5). An accumulation of DNA may be correlated with the increase in size of bacterial nuclei, examined by light microscopy (11). The DNA made during CM treatment appears to be functional (3). The ribonucleic acid (RNA) made during exposure of bacteria to CM is increased in amount (8, 11) and appears to be abnormal in certain respects, including its localization within the cell (2, 6). Recent studies of CM-treated bacteria have shown the accumulation of messenger RNA (12, 20, 24), and a family of small ribonucleoproteins (18*S* and 23*S*) named CM-particles (19, 25). The latter vary in size and in the ratios of RNA to protein, thus differing from the ribosomes of normal cells (19, 25), but the RNA present in CM-particles appears to be identical to normal ribosomal RNA (19, 25). Indeed, evidence has been presented which indicates that, upon removal of CM, bacteria transform CM-particles into ribosomes (13, 14, 23, 31). The protein of CM-particles is presumably derived from a pool of ribosomal protein subunits that are formed before the addition of CM (14, 18).

In view of the foregoing observations, it was decided to examine by electron microscopy *Escherichia coli* treated with CM in order to determine whether there were structural lesions that might correspond to the chemical changes. Previous studies of *E. coli* doubly fixed in glutaraldehyde and osmium tetroxide (21) revealed excellent preservation of cytoplasmic details; accordingly, this method of fixation was employed.

MATERIALS AND METHODS

The bacterial strain (*E. coli* C600) and the medium (HA) used in this study have been described, as have the procedures for determining the viability and turbidity of treated bacteria (26, 28).

Bacteria in medium HA were brought to the exponential growth phase (ca. 2×10^8 cells per ml), at which time portions of the culture were distributed into flasks containing CM, azauracil, hydroxyurea, or sodium azide (final concentrations, 40 μg/ml, 300 μg/ml, 0.2 M, and 0.01 M, respectively) or a mixture of these compounds. The cells were incubated at 37 C with aeration, and portions of the cultures were removed at intervals for determination of viability and turbidity and for electron microscopic examination.

In those experiments in which CM was removed, the bacteria were washed several times with sterile saline and resuspended to their original density in warm medium HA.

The preparation of specimens for electron microscopy followed the procedure outlined previously (21). The bacteria were fixed for 3 hr in 1% glutaraldehyde, washed thoroughly, fixed for 3 hr in osmium tetroxide, dehydrated in ethyl alcohol, and embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate.

Azauracil was obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y.; CM from Parke, Davis and Co., Detroit, Mich.; hydroxyurea from Nutritional Biochemicals Corp., Cleveland, Ohio; and sodium azide from Fisher Scientific Co., New York, N.Y. Solutions of hydroxyurea were always freshly prepared.

RESULTS

Figures 1–3 illustrate normal bacteria, showing the average length of the cells, the disposition of ribosomes, and variation in the appearance of nuclear material, the last being characterized by translucent areas containing fine and coarse, often branched, filaments. Figures 1–26 are reproduced at the same magnification (× 40,000) to facilitate comparison.

One hour after addition of CM (Fig. 4–6), the nuclear material had begun to aggregate at the center of the bacteria, and the cytoplasm was more finely granular, compared to the controls, suggesting that the normal ribosomes had become disassembled. It is of interest that bacteria presumed to be in the process of binary fission when the drug was added had two foci at which nuclear material localized (Fig. 6), although bacterial division did not appear to proceed.

By 2 hr (Fig. 7–9), some bacteria had become elongated (Fig. 9); others developed scattered cytoplasmic vacuoles devoid of limiting membranes (Fig. 7). There was frequently a slight retraction of the cytoplasmic membrane from the cell wall (Fig. 7). Examination of the bacteria shown in Fig. 5 and 9 reveals that cytoplasm protrudes into the nuclear matrix, the appearance of an island of cytoplasm (Fig. 6–8) probably reflecting the angle and level of sectioning. In rare instances, a small cluster of dense granules appeared within a cytoplasmic vacuole (see arrow in Fig. 8). By 4 hr, the clusters had become larger and more numerous, although in any given bacterium there were seldom more than two. They tended to be located at the ends of the cell (Fig. 10). By 23 hr (Fig. 11–13), the aggregates were still larger and the granules more uniform in size. The nuclear region was nearly devoid of cytoplasmic protrusions. In addition, there were now collections of amorphous, electron-dense material within, and at the periphery of, the nuclear matrix. Not infrequently, greatly elongated bacteria were observed (Fig. 12).

With removal of CM, the bacteria underwent sequential, though not synchronous, changes in the process of reversion to the normal state. The first to be observed (in samples taken at 83 min) were beginning redistribution of the nuclear matrix and disappearance of the dense amorphous material (Fig. 14 and 15). By 130 min, the nuclear material was nearly normal in appearance and distribution (Fig. 16–19). Analogous changes also occurred in cells presumed to have been arrested in division by the addition of CM (Fig. 18). When aggregates of cytoplasmic granules were transected, clearing was observed at the periphery of the cluster (Fig. 19). By 145 min (Fig. 20–23), the aggregates of granules had disappeared and ribosomes were again apparent in the cytoplasm. Attempted division apparently had begun but the process was abnormal. There were tortuous, irregular infoldings of the plasma membrane unaccompanied by ingrowth of the cell wall. Bacteria rarely were encountered at stages of normal cleavage or fission. By 190 min (Fig. 24–26), normal bacterial division finally was observed, but simultaneously bacteria were also seen which exhibited the changes shown to occur at preceding intervals. Presumably, such cells had died in the course of the experiment and had maintained the structural abnormalities that were present when metabolism ceased.

Fine structure could be seen with greater clarity at higher magnification, which revealed several unusual features. Two hours after addition of CM, the abnormal cytoplasmic granules were not uniform in appearance. In Fig. 27, for example, there is a small cluster of granules which differ in size. Above and to the right, convolutions of the plasma membrane lie at various angles to the plane of section. (Such convolutions are illustrated more clearly in Fig. 29.) In Fig. 28, the granules are sparse in both polar aggregates, and thin filaments are visible. Four hours after addition of CM, the clusters of granules were larger and the granules themselves showed marked variation in size and shape (Fig. 29 and 30). It is of interest that, within 4 hr, reduplication of the plasma membrane was frequently encountered in close proximity to the clusters of granules. Such reduplication is evident at two sites near the uppermost aggregate in Fig. 29. A striking example of reduplicated membranes is also seen in Fig. 30. In view of the unlikelihood of such membranes lying perpendicular to the plane of section and hence appearing well defined, their frequent presence would suggest that they were very numerous indeed. Laminated membranes in association with the clusters of abnormal granules were not as commonly encountered at later intervals after drug addition, but they again become numerous upon removal of the CM. Figure 31,

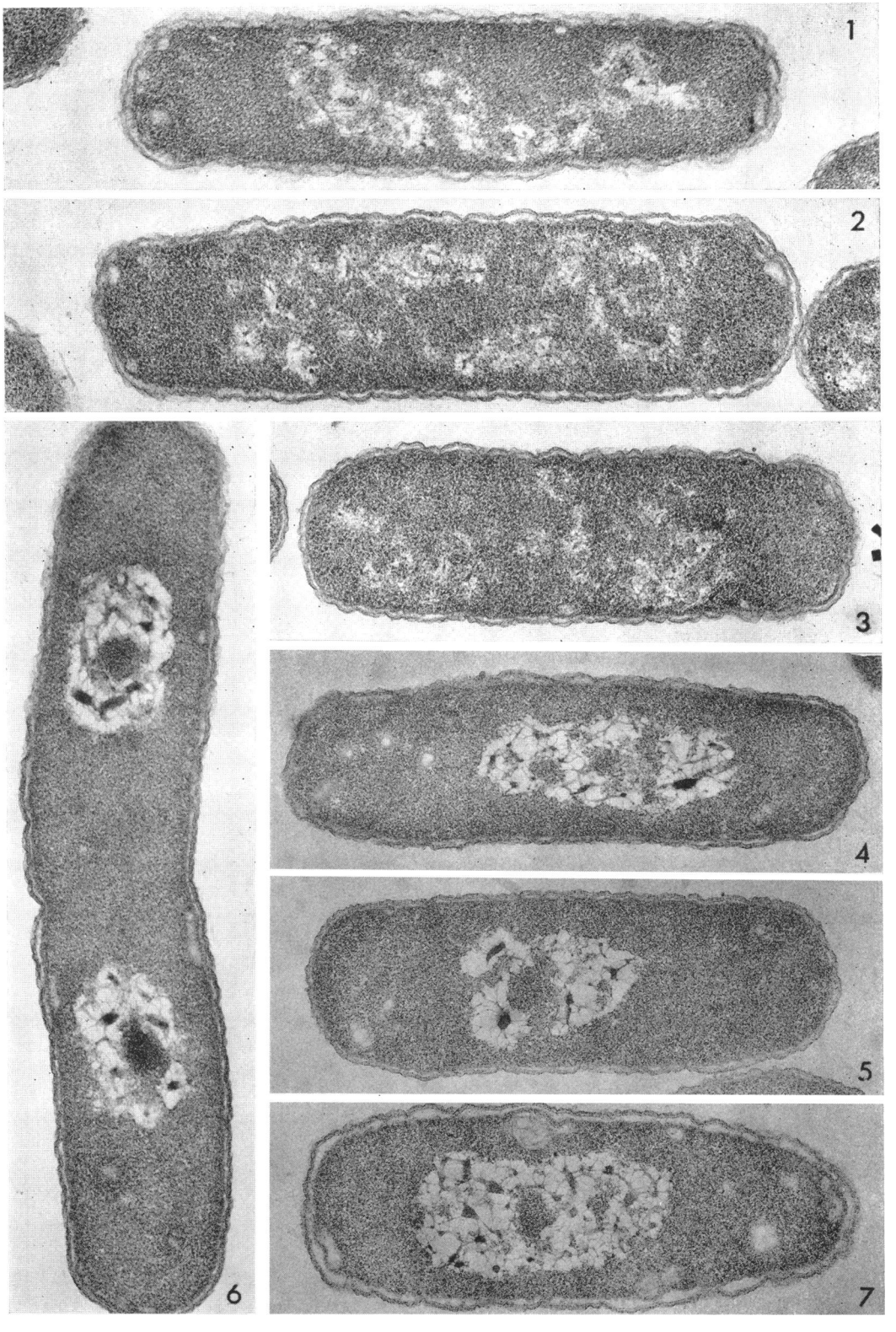


FIG. 1–3. *Untreated, control bacteria. Fig. 1–26, × 40,000.*
FIG. 4–6. *Chloramphenicol, 1 hr.*
FIG. 7. *Chloramphenicol, 2 hr.*

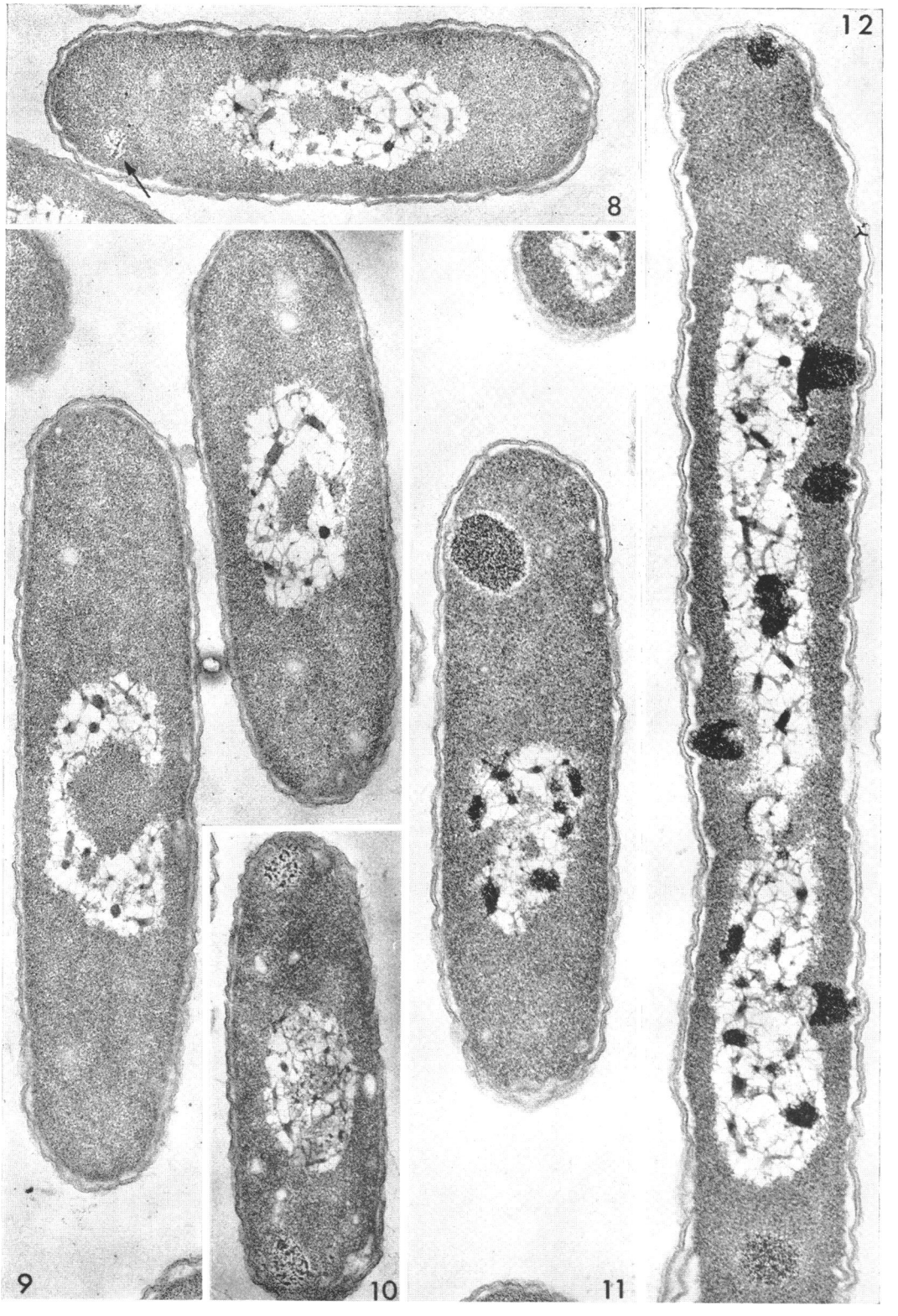


FIG. 8 and 9. *Chloramphenicol, 2 hr.*
FIG. 10. *Chloramphenicol, 4 hr.*
FIG. 11 and 12. *Chloramphenicol, 23 hr.*

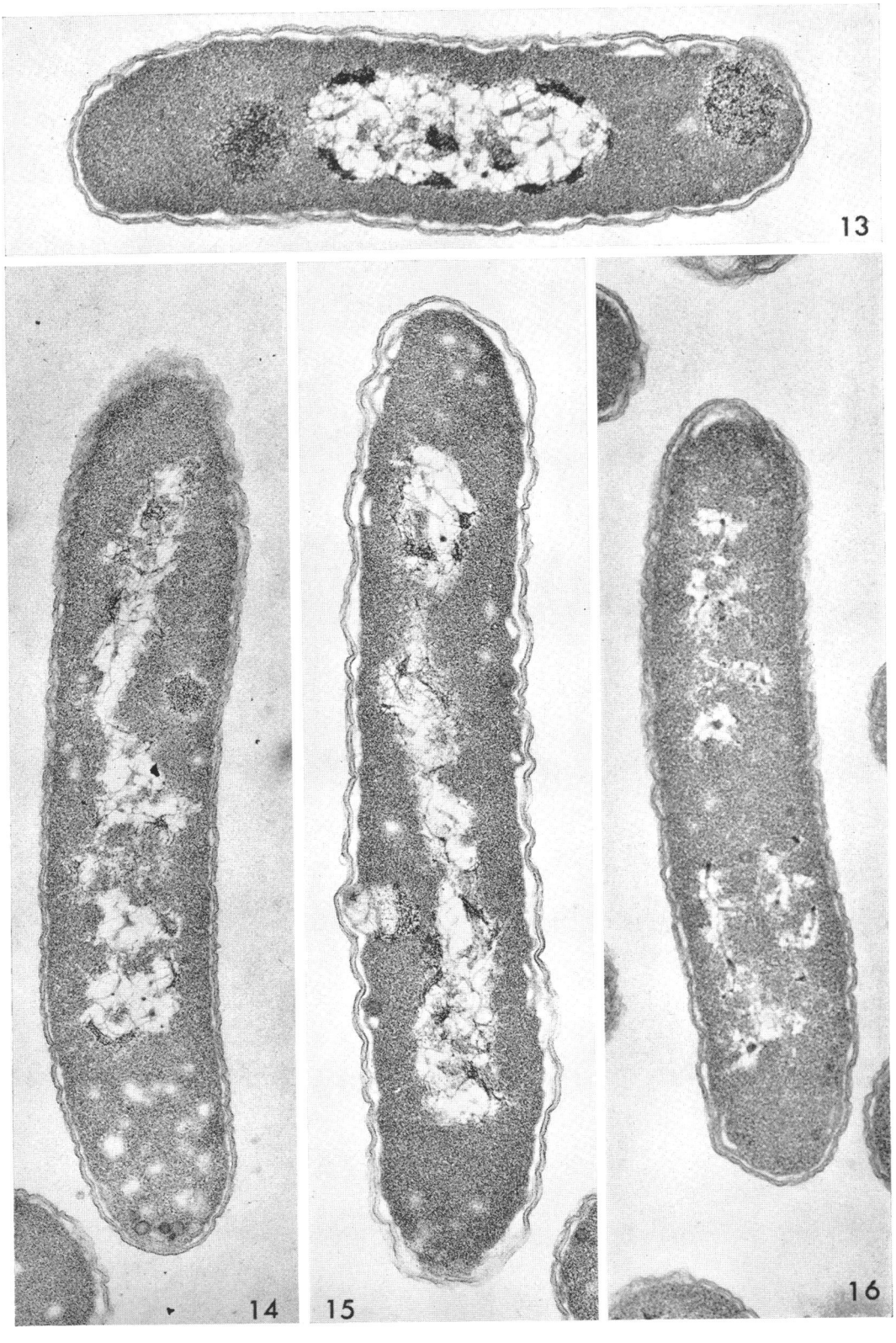


FIG. 13. *Chloramphenicol, 23 hr.*
FIG. 14 and 15. *Cells 83 min after removal of chloramphenicol.*
FIG. 16. *Cell 130 min after removal of chloramphenicol.*

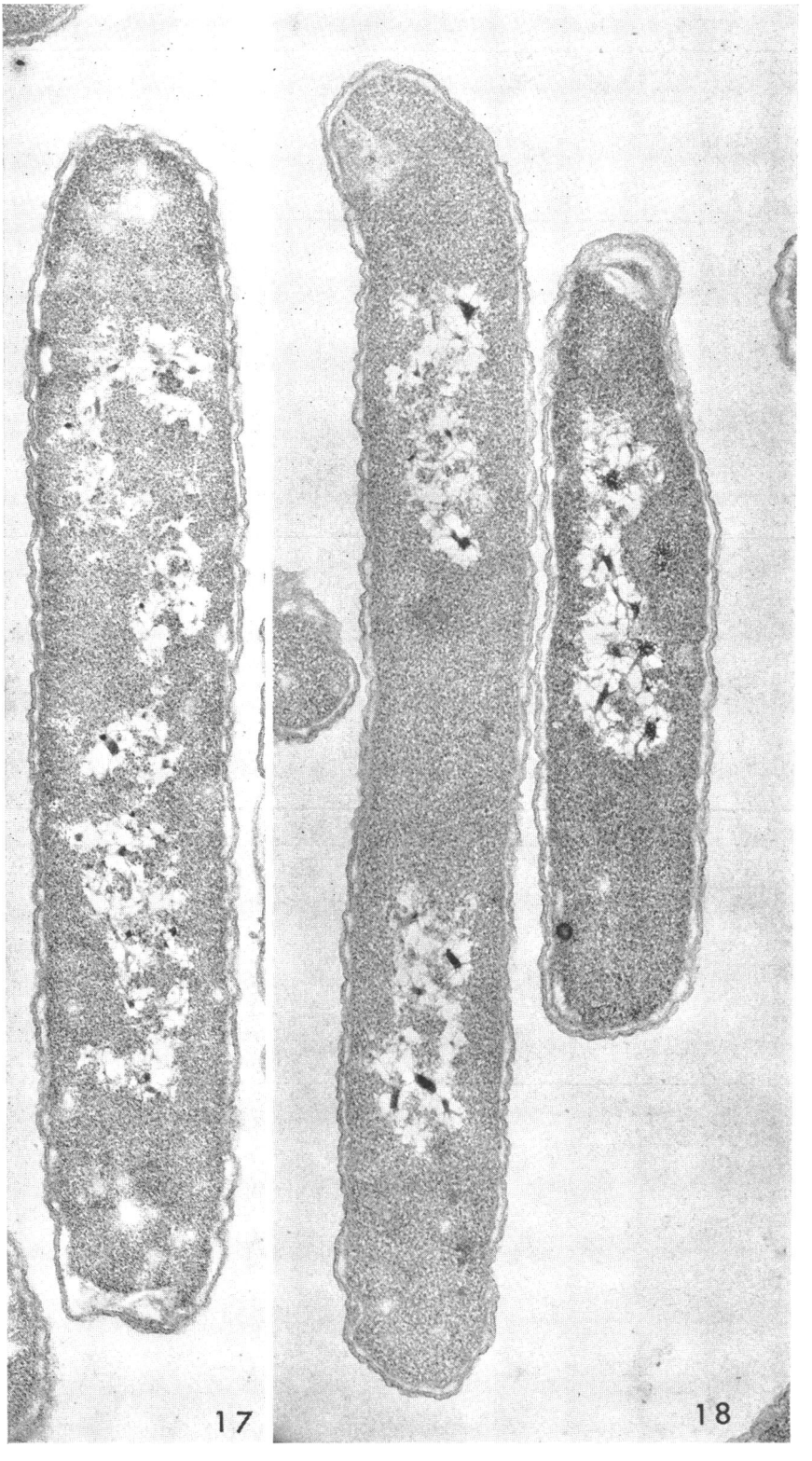


FIG. 17 and 18. *Cells 130 min after removal of chloramphenicol.*

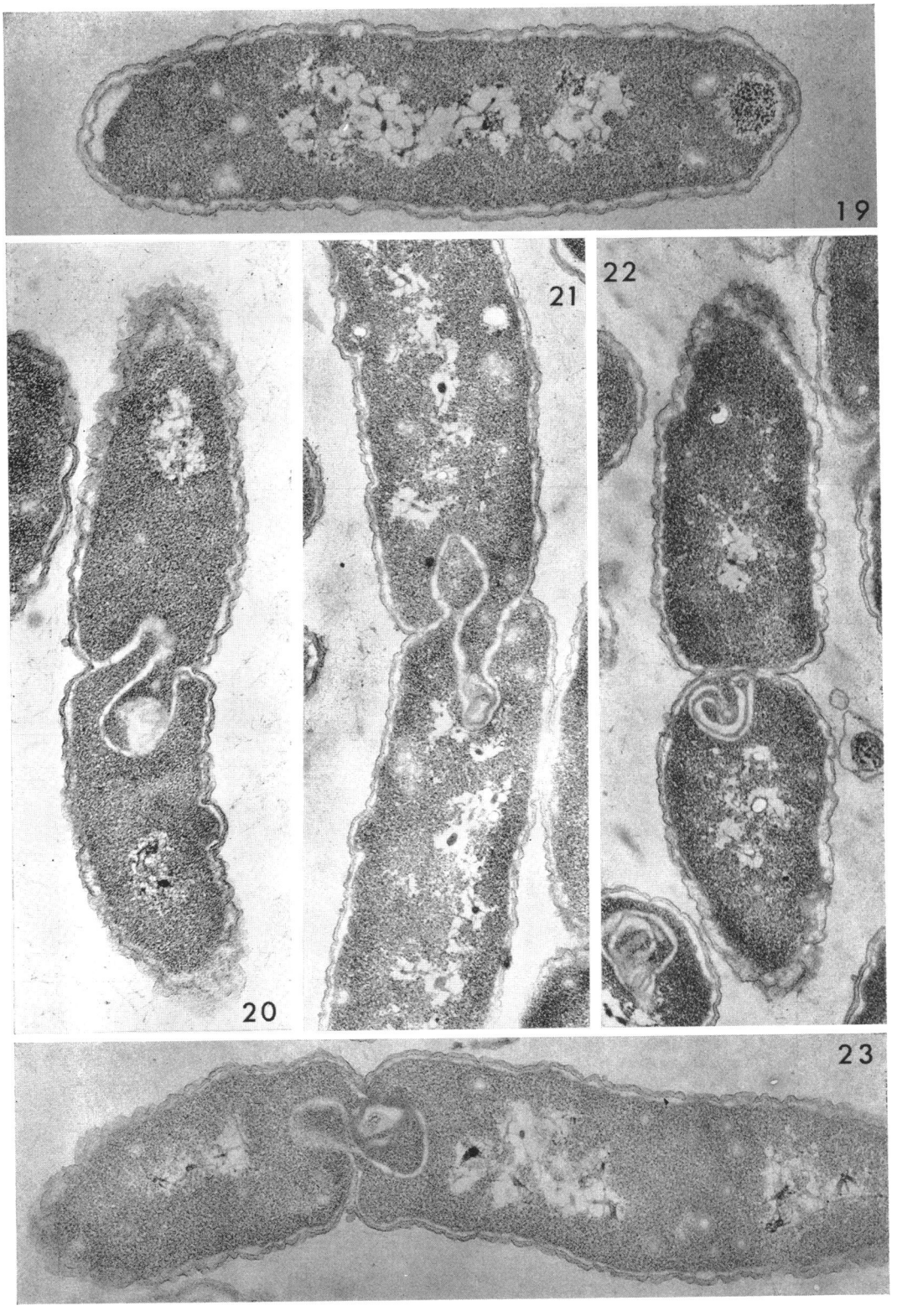


FIG. 19. *Cell 130 min after removal of chloramphenicol.*
FIG. 20–23. *Cells 145 min after removal of chloramphenicol.*

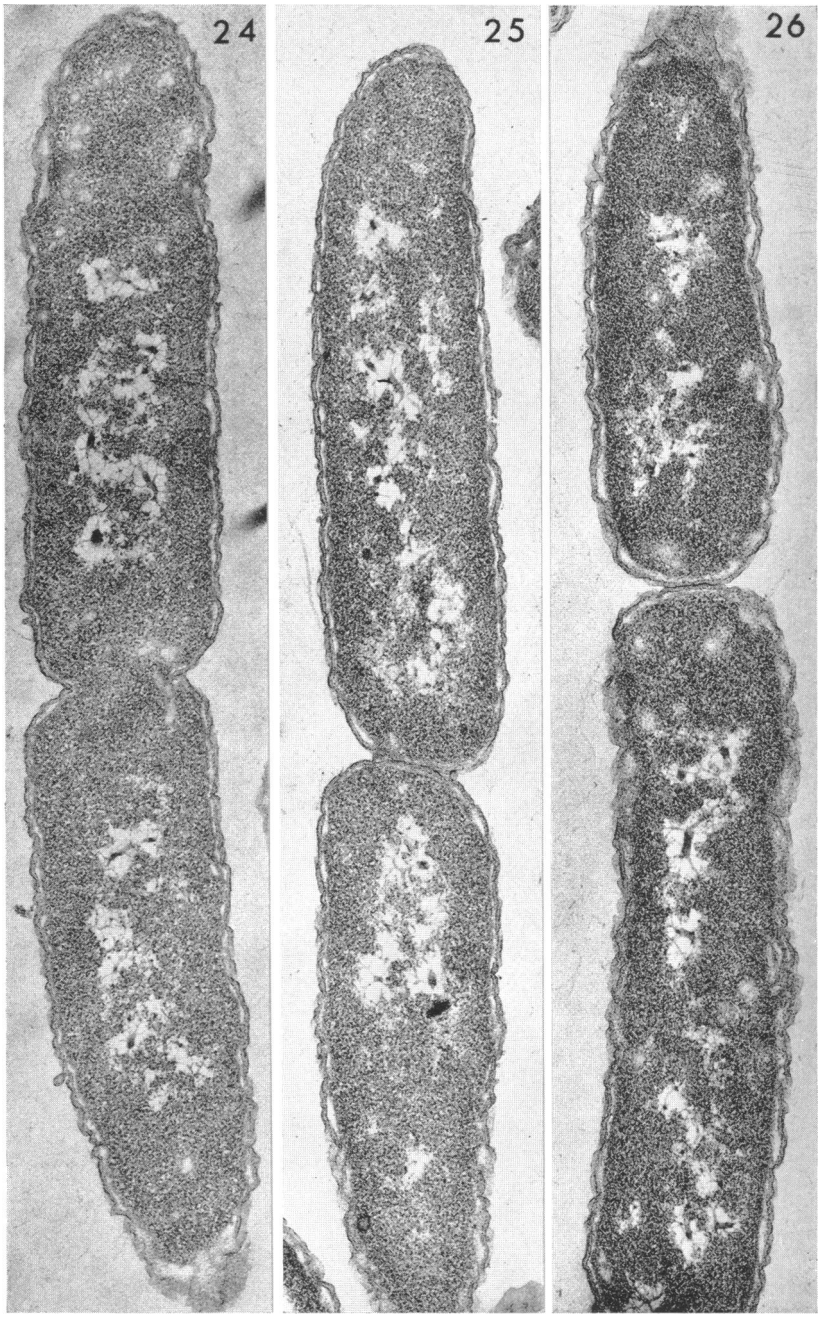


FIG. 24–26. *Cells 190 min after removal of chloramphenicol.*

for example, shows a bacterium 130 min after drug removal. Laminated membranes, this time on the inner aspect of the aggregate of abnormal granules, are clearly seen.

Figure 32 illustrates a bacterium 23 hr after addition of CM. The clusters of abnormal cytoplasmic granules and dense amorphous perinuclear material are evident. (It will be noted that the granules are more closely packed and more uniform in size than those shown in Fig. 29 and 30.) Retraction of the plasma membrane from the cell wall is also seen. For comparison of

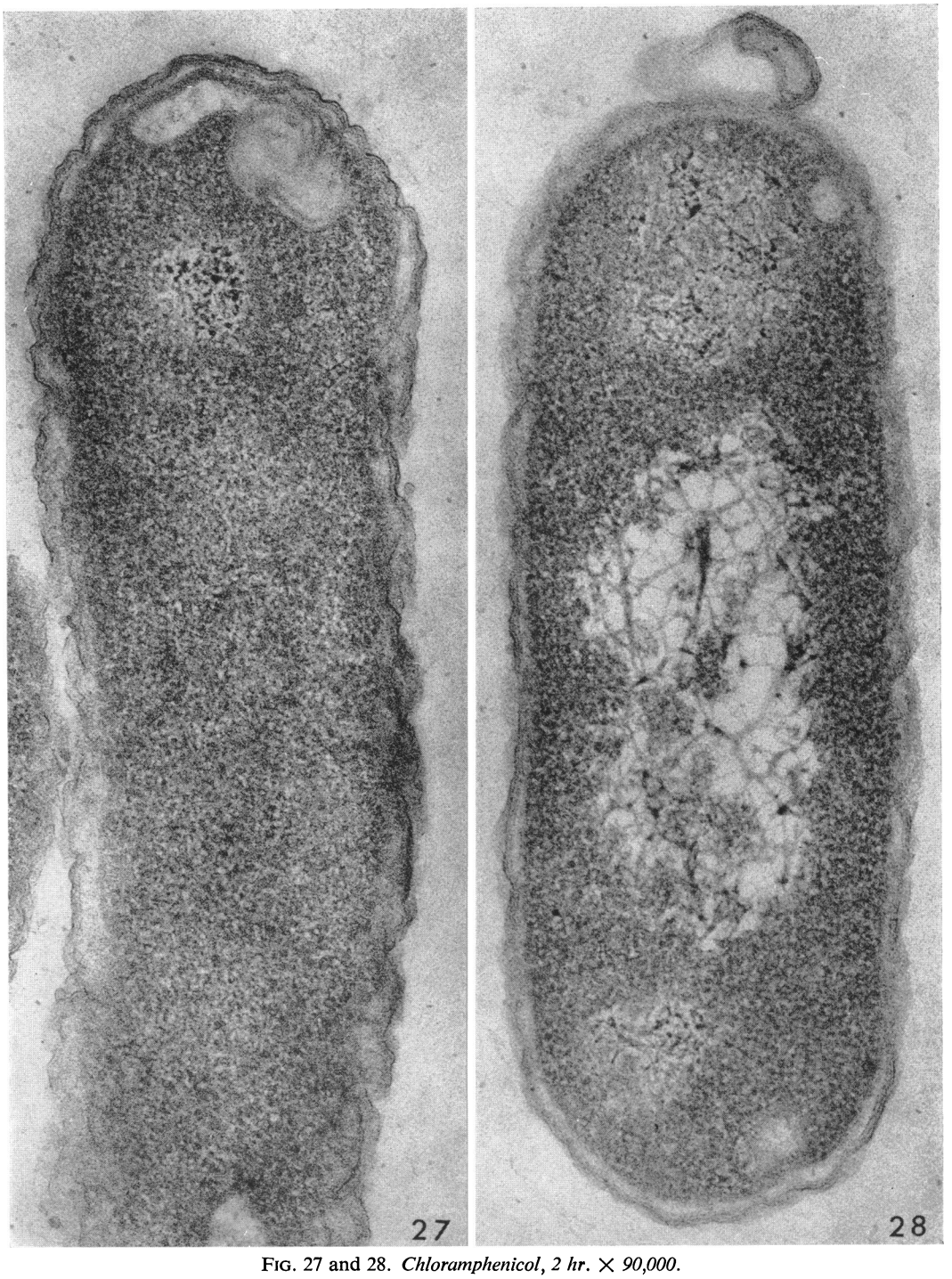


FIG. 27 and 28. *Chloramphenicol, 2 hr.* × *90,000.*

the ribosomes, a normal bacterium undergoing division is seen on the right (Fig. 33). (Unfortunately, to show the entire bacterium, a different magnification had to be used.) Abnormal or abortive attempts at division are seen in Fig. 34–36 (145 min after removal of CM). In Fig. 34, the plasma membrane, although slightly oblique to the section and hence not clearly defined,

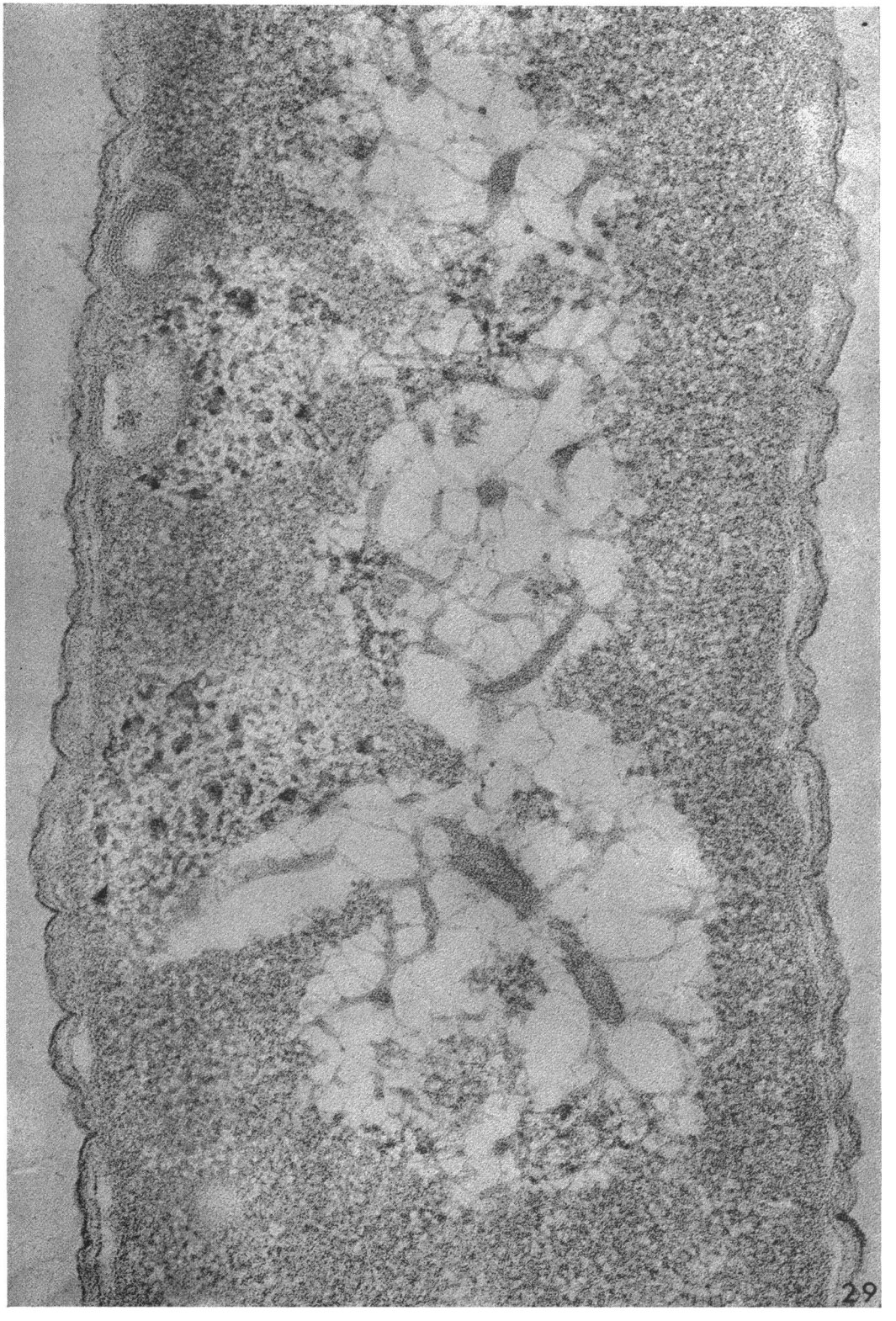
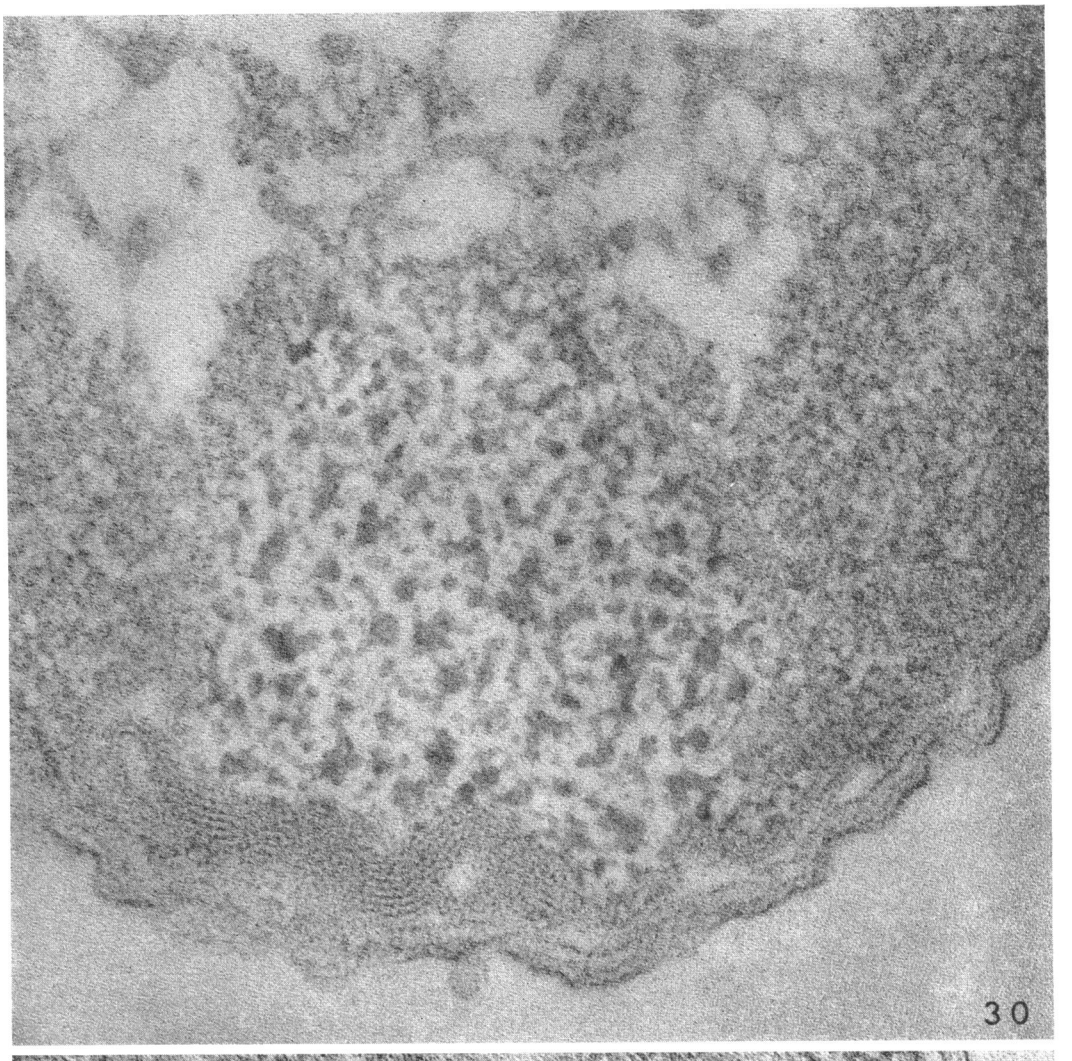


FIG. 29. *Chloramphenicol, 4 hr.* × *155,000.*



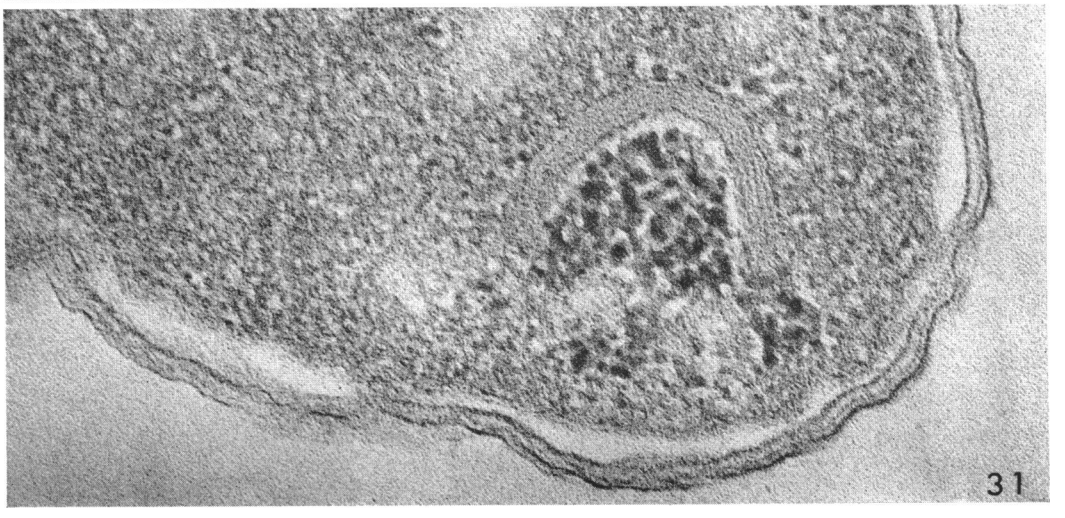


FIG. 30. *Chloramphenicol, 4 hr.* × *260,000.*
FIG. 31. *Cell 130 min after removal of chloramphenicol.* × *176,000.*

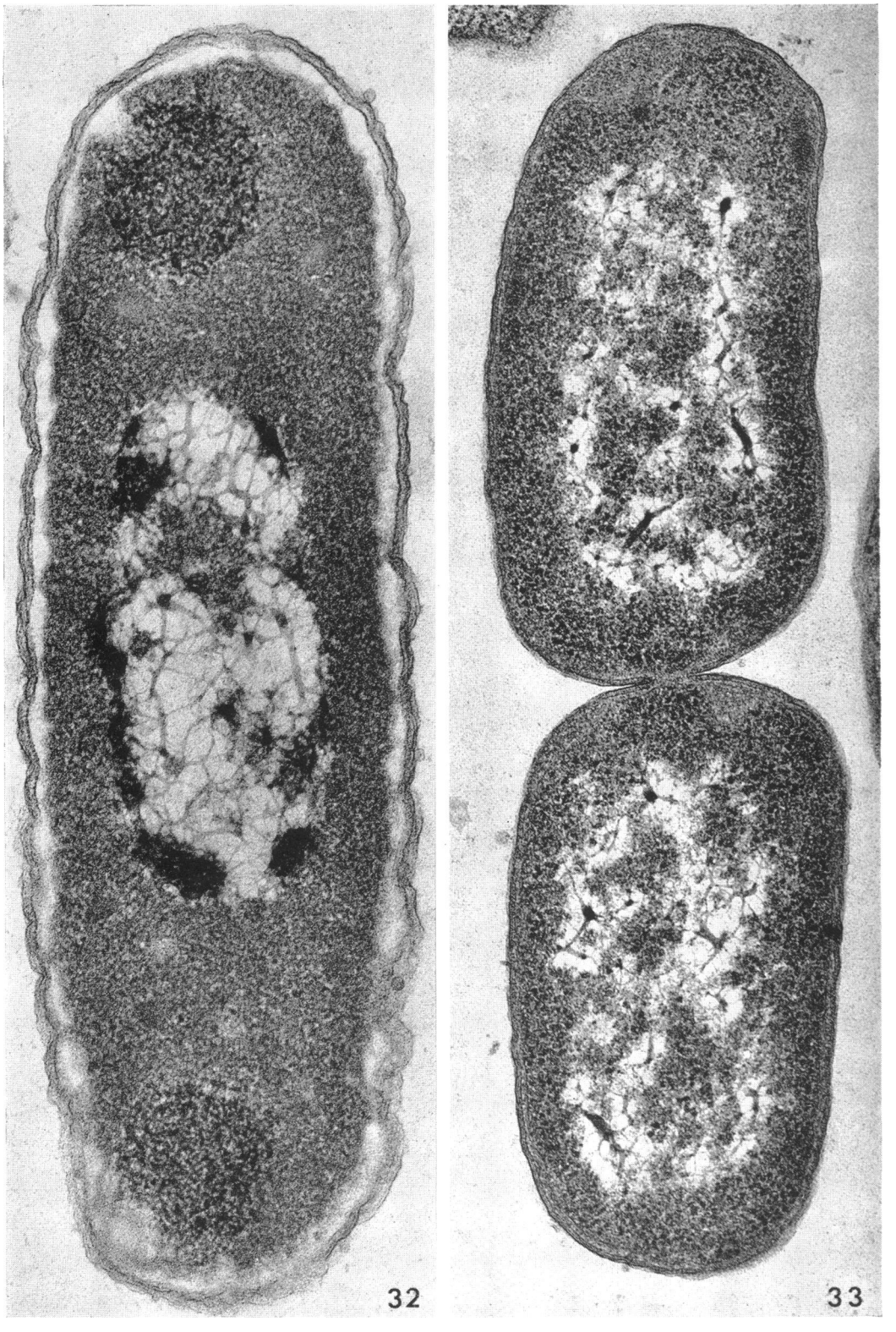


FIG. 32. *Chloramphenicol, 23 hr.* × *57,000.*
FIG. 33. *Untreated bacterium.* × *90,000.*

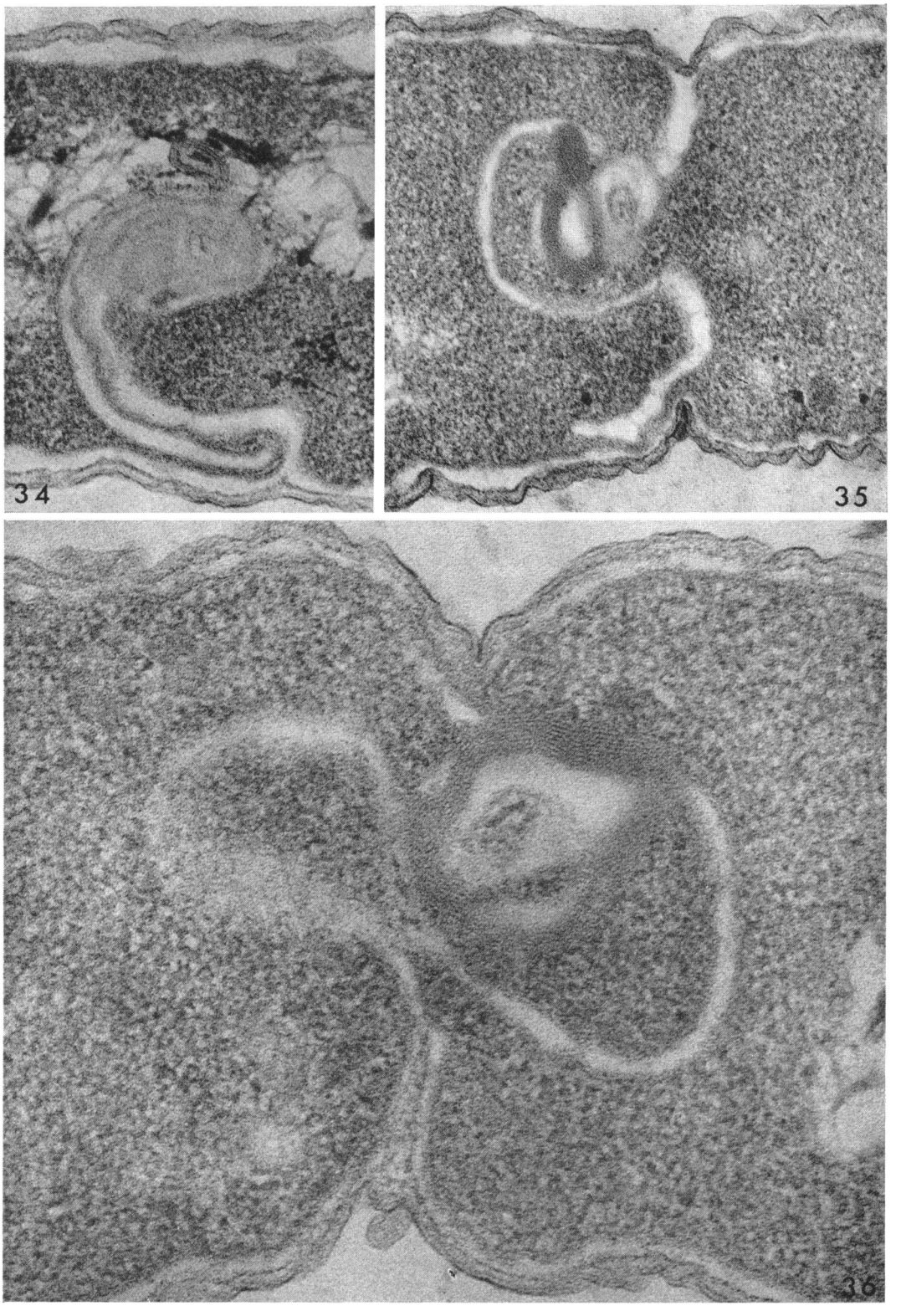


FIG. 34 and 35. *Cells 145 min after removal of chloramphenicol.* × *103,000.*
FIG. 36. *Cell 145 min after removal of chloramphenicol.* × *164,000.*

appears to have become convoluted and to extend into the nuclear matrix. The cell wall is not altered. Figures 35 and 36 show complex infoldings of the plasma membrane, together with the presence of laminated membranes. It is not clear whether the latter are reduplications of the plasma membrane or are laid down de novo. In comparison to cells undergoing normal division (Fig. 33), the walls of bacteria such as these do not grow inward in an orderly fashion, and thus lead to cleavage of the cell.

To ascertain the nature of the various structures seen in CM-treated bacteria, cells were treated with sodium azide (an inhibitor of energy metabolism), azauracil (an inhibitor of RNA synthesis), and hydroxyurea (an inhibitor of DNA synthesis). The biological effects of these inhibitors are listed in Table 1. CM alone is bacteriostatic, as is azauracil (27). Hydroxyurea alone is bactericidal, whereas CM prevents the bactericidal action of hydroxyurea (27).

Cells treated with both CM and sodium azide closely resembled normal bacteria. Bacteria treated with CM and azauracil contained clusters of granules in the cytoplasm but lacked the electron-dense amorphous material at the periphery of the nuclear matrix. Central aggregation of nuclear material was also observed, but the phenomenon was less consistently encountered than in bacteria treated with CM alone. Bacteria treated only with azauracil showed no clusters of dense granules, and the nuclear material was normally distributed. Bacteria exposed simultaneously to hydroxyurea and CM contained the electron-dense, amorphous material as well as the clusters of dense granules. (The electron-microscopic appearance of cells treated with hydroxyurea alone showed complex changes, which will form the subject of a future report.) Bacteria treated with CM, hydroxyurea, and azauracil simultaneously had nuclei that were normal in appearance. Clusters of cytoplasmic granules were encountered but they were not numerous; there was also extensive vacuolization of the cytoplasm.

TABLE 1. *Effect of various inhibitors on the growth of Escherichia coli*[a]

Expt	Time	Additions	Turbidity	Viable bacteria/ml
	hr			
I	0	—	0.286	1.6×10^8
	23	None	1.5	2.3×10^9
	23	CM, 40 μg/ml	0.412	1.8×10^8
	23	Azauracil, 300 μg/ml	1.2	4.3×10^8
	23	CM + azauracil	0.390	1.3×10^8
II	0	—		2.8×10^8
	24	None		4.4×10^9
	24	Hydroxyurea, 0.2 M		5.0×10^4
	24	CM, 40 μg/ml		3.0×10^8
	24	CM + hydroxyurea		1.0×10^8
III	0	—		2.5×10^8
	24	None		2.7×10^9
	24	CM, 40 μg/ml		3.3×10^8
	24	NaN_3, 0.01 M		2.1×10^8
	24	CM + NaN_3		1.9×10^8
	24	CM + azauracil + hydroxyurea		1.7×10^8

[a] Bacteria (*E. coli* C600) in medium HA were brought to the exponential growth phase, at which time portions were divided into flasks containing measured amounts of the various drugs. At zero-time and at the end of 23 or 24 hr, the viability and turbidity of the cultures were determined and representative portions of the bacteria were processed for electron microscopy.

DISCUSSION

The morphological changes seen in the electron microscope can be correlated with the biochemical events that have been described in CM-treated bacteria. Thus, the cytoplasmic granules may well be the CM-particles. Their formation, which is not dependent on DNA and RNA synthesis (they occur in bacteria treated with either hydroxyurea or azauracil), is consistent with the hypothesis that they are derived from pre-existing molecules (14, 18, 25). The variation in size and shape of the granules revealed by the electron micrographs would be expected on the basis of existing chemical and physicochemical data (14). In view of the fact that laminated membranes, which appeared to result from reduplication of the plasma membrane, were frequently encountered in close proximity to the granules, particularly at times when the granules were either being formed from (Fig. 29 and 30) or assembled into (Fig. 31) ribosomes, leads to the conclusion that both processes depend on the presence of these laminated membranes. The membranes appear to differ from the septate form encountered in *E. coli* after magnesium repletion (22) and from the structures encountered by Schnaitman and Greenawalt (30) in one strain of *E. coli*.

It has been suggested that DNA synthesis occurs at the cell membrane, which becomes laminated and infolded to form mesosomes (7, 32). These structures are uncommon in *E. coli*, having been reported only by Kaye and Chapman (16) and by Ryter and Jacob (29). In the present study, extensive examination of untreated bacteria rarely revealed reduplication of the plasma membrane. Nevertheless, the nuclear matrix was fre-

quently observed in close proximity to the plasma membrane (Fig. 1–3). In normal bacteria, the DNA of the nuclear matrix presumably is synthesized close to, or on, the peripheral membrane of the cell and migrates inward, with resulting dispersal of the nuclear material in the cytoplasm. When bacteria are treated with CM, the cycle of DNA synthesis is completed and is followed by detachment of DNA from sites near the cell membrane and by central aggregation. Kellenberger, Ryter, and Séchaud (17), as well as Franklin and Granboulan (9), observed this central location of the nuclear matrix shortly after cells were treated with CM. (They described it as the "vesicular form of the nucleoid," a phrase which is not entirely clear.) On the basis of the foregoing model, it could be predicted that, after CM was removed and before cellular replication was resumed, the DNA would assume its former distribution. This indeed was found to occur.

The amorphous, electron-dense material is undoubtedly RNA, since it was not found in cells treated with azauracil, which blocks RNA synthesis. Its presence near the DNA of the nuclear matrix agrees with "chromatin" localization of the RNA, which has been described in CM-treated bacteria (6). Presumably, this material is actually the messenger RNA, which is known to accumulate (12, 20, 24).

It is of interest that the CM-induced morphological changes are dependent upon energy metabolism (inhibited by sodium azide) and that they are independent of DNA synthesis (not prevented by hydroxyurea in combination with CM). Elongation of the bacteria and delay in the recovery from the effects of CM treatment have been described by other investigators (1, 4). The presence of infolded reduplications of the plasma membrane is a prominent feature of the abortive attempts at cell division that follow removal of CM (Fig. 20–23, 34–36). This phenomenon, at least as observed in the present experiments, is not consistent with the hypothesis advanced by Fitz-James (7) and Van Iterson (32) that mesosomes are related to the formation of the cell wall.

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LITERATURE CITED

1. ALLISON, J. L., R. E. HARTMAN, R. S. HARTMAN, A. D. WOLFE, J. CIAK, AND F. E. HAHN. 1962. Mode of action of chloramphenicol. VII. Growth and multiplication of *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. **83**:609–615.
2. ARONSON, A. I., AND S. SPIEGELMAN. 1961. On the nature of the ribonucleic acid synthesized in the presence of chloramphenicol. Biochim. Biophys. Acta **53**:84–95.
3. BROCK, T. D. 1961. Chloramphenicol. Bacteriol. Rev. **25**:32–48.
4. BROCK, T. D., AND M. L. BROCK. 1961. RNA synthesis and degradation during antibiotic treatment and its relation to antibiotic-induced lag. J. Gen. Microbiol. **26**:393–404.
5. DRAKULIĆ, M., AND M. ERRERA. 1959. Chloramphenicol-sensitive DNA synthesis in normal and irradiated bacteria. Biochim. Biophys. Acta **31**:459–463.
6. EZEKIEL, D. H. 1961. Increase in ribonucleic acid in the bacterial chromatin body during chloramphenicol treatment. J. Bacteriol. **81**:319–326.
7. FITZ-JAMES, P. C. 1960. Participation of the cytoplasmic membrane in the growth and spore formation of bacilli. J. Biophys. Biochem. Cytol. **8**:507–528.
8. FRAENKEL, D. G., AND F. C. NEIDHARDT. 1961. Use of chloramphenicol to study control of RNA synthesis in bacteria. Biochim. Biophys. Acta **53**:96–110.
9. FRANKLIN, R. M., AND N. GRANBOULAN. 1966. Ultrastructure of *Escherichia coli* cells infected with bacteriophage R17. J. Bacteriol. **91**:834–848.
10. GALE, E. F., AND T. F. PAINE. 1951. The assimilation of amino-acids by bacteria. 12. The action of inhibitors and antibiotics on the accumulation of free glutamic acid and the formation of combined glutamate in *Staphylococcus aureus*. Biochem. J. **48**:298–301.
11. HAHN, F. E., M. SCHAECHTER, W. S. CEGLOWSKI, H. E. HOPPS, AND J. CIAK. 1957. Interrelations between nucleic acid and protein biosynthesis. I. Synthesis and fate of bacterial nucleic acids during exposure to, and recovery from the action of chloramphenicol. Biochim. Biophys. Acta **26**:469–476.
12. HAHN, F. E., AND A. D. WOLFE. 1961. Mode of action of chloramphenicol. VIII. Resemblance between labile chloramphenicol-RNA and DNA of *Bacillus cereus*. Biochem. Biophys. Res. Commun. **6**:464–468.
13. HOROWITZ, J., AND D. C. HILLS. 1966. Evidence for the direct conversion of chloramphenicol particles into ribosomes in *Escherichia coli*. Biochim. Biophys. Acta **123**:416–419.
14. HOSOKAWA, K., AND M. NOMURA. 1965. Incomplete ribosomes produced in chloramphenicol- and puromycin-inhibited *Escherichia coli*. J. Mol. Biol. **12**:225–241.

15. JULIAN, G. R. 1965. [^{14}C] lysine peptides synthesized in an *in vitro Escherichia coli* system in the presence of chloramphenicol. J. Mol. Biol. **12**:9–16.
16. KAYE, J. J., AND G. B. CHAPMAN. 1963. Cytological aspects of antimicrobial antibiosis. III. Cytologically distinguishable stages in antibiotic action of colistin sulfate on *Escherichia coli*. J. Bacteriol. **86**:536–543.
17. KELLENBERGER, E., A. RYTER, AND J. SÉCHAUD. 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phase DNA as compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. **4**:671–678.
18. KURLAND, C. G., AND O. MAALØE. 1962. Regulation of ribosomal and transfer RNA synthesis. J. Mol. Biol. **4**:193–210.
19. KURLAND, C. G., M. NOMURA, AND J. D. WATSON. 1962. The physical properties of the Chloromycetin particles. J. Mol. Biol. **4**:388–394.
20. MANDEL, L. R., AND E. BOREK. 1962. Ribonucleic acid synthesis under relaxed control. Biochem. Biophys. Res. Commun. **9**:11–17.
21. MARGARETTEN, W., C. MORGAN, H. S. ROSENKRANZ, AND H. M. ROSE. 1966. Effect of hydroxyurea on virus development. I. Electron microscopic study of the effect on the development of bacteriophage T4. J. Bacteriol. **91**:823–833.
22. MORGAN, C., H. S. ROSENKRANZ, B. CHAN, AND H. M. ROSE. 1966. Electron microscopy of magnesium-depleted bacteria. J. Bacteriol. **91**:891–895.
23. NOMURA, M., AND K. HOSOKAWA. 1965. Biosynthesis of ribosomes: fate of chloramphenicol particles and of pulse-labeled RNA in *Escherichia coli*. J. Mol. Biol. **12**:242–265.
24. NOMURA, M., K. OKAMOTO, AND K. ASANO. 1962. RNA metabolism in *Escherichia coli* infected with bacteriophage T4. Inhibition of host ribosomal and soluble RNA synthesis by phage and effect of Chloromycetin. J. Mol. Biol. **4**:376–387.
25. NOMURA, M., AND J. D. WATSON. 1959. Ribonucleoprotein particles within Chloromycetin-inhibited *Escherichia coli*. J. Mol. Biol. **1**:204–217.
26. ROSENKRANZ, H. S., AND A. J. BENDICH. 1964. Studies on the bacteriostatic action of hydroxylamine. Biochim. Biophys. Acta **87**:40–53.
27. ROSENKRANZ, H. S., AND H. S. CARR. 1966. Studies with hydroxyurea. II. Prolonged exposure of *Escherichia coli* to hydroxyurea. J. Bacteriol. **92**:178–185.
28. ROSENKRANZ, H. S., H. S. CARR, AND H. M. ROSE. 1965. Phenethyl alcohol. I. Effect on macromolecular synthesis of *Escherichia coli*. J. Bacteriol. **89**:1354–1369.
29. RYTER, A., AND F. JACOB. 1966. Étude morphologique de la liaison du noyau à la membrane chez *E. coli* et chez les protoplastes de *B. subtilis*. Ann. Inst. Pasteur **110**:801–812.
30. SCHNAITMAN, C., AND J. W. GREENAWALT. 1966. Intracytoplasmic membranes in *Escherichia coli*. J. Bacteriol. **92**:780–783.
31. SPIRIN, A. S. 1963. *In vitro* formation of ribosome-like particles from CM particles and protein. Cold Spring Harbor Symp. Quant. Biol. **28**:267–268.
32. VAN ITERSON, W. 1961. Some features of remarkable organelle in *Bacillus subtilis*. J. Biophys. Biochem. Cytol. **9**:183–192.
33. WEBER, M. J., AND J. A. DEMOSS. 1966. The inhibition by chloramphenicol of nascent protein formation in *E. coli*. Proc. Natl. Acad. Sci. U.S. **55**:1224–1230.