THE FINE STRUCTURE OF THE RIBONUCLEOPROTEIN
IN BACTERIAL CYTOPLASM

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The physical organization of bacterial ribosomes has been determined mainly from the study of purified fractions of disintegrated cells. In sections of cells from the exponential phase of growth, the cytoplasmic components are usually so densely packed that neither their shape nor their size is readily distinguishable. As a consequence, little work has been reported correlating biochemical information with bacterial morphology in sections at good resolution.

Bacteria are one of the richest sources of ribonucleoprotein. According to Tissières et al. (21), ribonucleoprotein constitutes 30% of the dry weight in rapidly growing *Escherichia coli* cells, 63% being RNA and 37% protein. In the electron microscopic picture of bacterial cytoplasm, therefore, we may expect a great deal of the fine structure to consist of RNA. It is RNA that endows growing bacteria with their well known affinity for basic dyes.

It has been inferred from centrifugation studies that, in bacteria, RNA is predominantly contained in particles with a sedimentation constant of 70 S and that the synthesis of protein is carried out in these particles. These particles are described as consisting of two unequal subunits, 30 and 50 S; the long and short axes of the 70-S particles have been estimated from electron micrographs to be 200 by 170 A (5), or about 180 to 200 by 170 A (9, 23), or 190 by 160 A (18). But in electron micrographs of bacterial cytoplasm, we have not observed particles of such large size and consisting of these subunits.

When gentler methods of cell rupture were subsequently applied, the picture of the bacterial cell as a bag containing hundreds of independent ribosomes was modified by the discovery of polyribosomes or ergosomes (4, 16, 17, 22). These multiple aggregates of 70-S ribosomes, thought to be held together by a simple thread of messenger RNA, now appear to be the most active participants in protein synthesis. Recently it has been suggested that in bacteria, comparable to the situation in animal (13) and plant cells, the polysomes are arranged on intracellular membranes, and perhaps on the plasma membrane (17, 19, 3, 7, 8, 14, 20, 6, 12).

From electron micrographs, it has been derived that bacteria do not have a limiting membrane between their nucleoplasm and cytoplasm. Yet nothing really is known morphologically about the relationship between the DNA fibers in the nucleoplasm and the protein-synthesizing system in the cytoplasm. The closest representation of the natural condition probably is shown in the electron micrographs of the DNA-ribosome complex formed during protein synthesis in cell-free extracts of *E. coli* (2, 1). Here clusters of particles are revealed which could be the 70-S ribosomes aggregated about long fibers, 15 to 20 A wide and presumably the DNA. From these long fibers, strands branch off which could be the RNA linking the DNA to ribosomes.

We are making a high resolution study of bacterial cytoplasm under as natural conditions as possible, studying organisms whose structure is
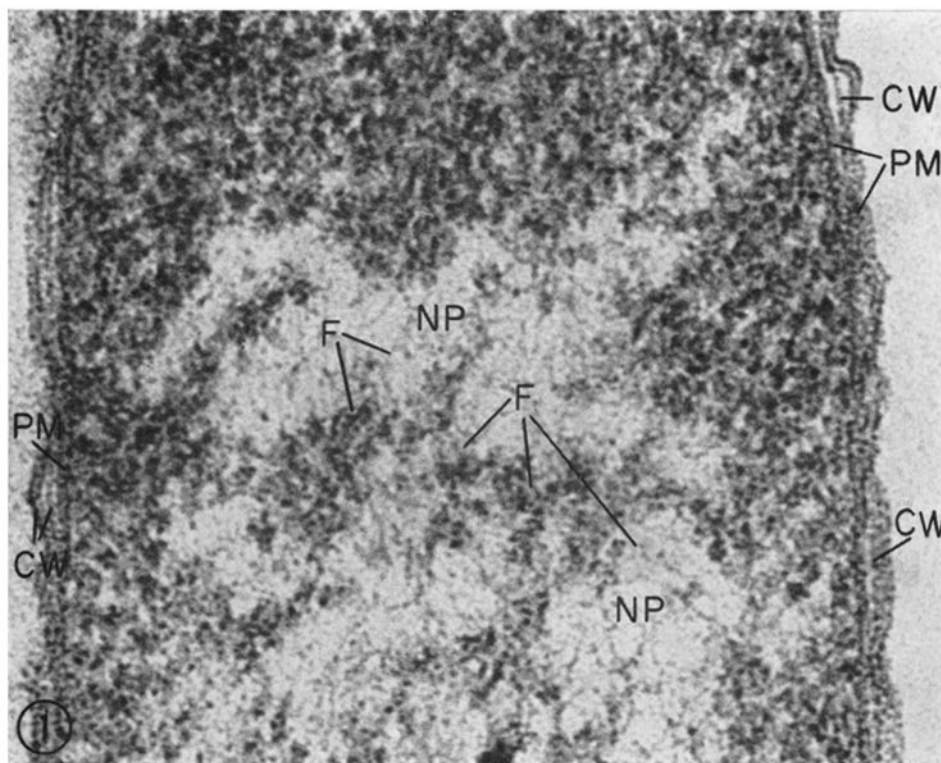
FIGURE 1 From a thin section of *E. coli* B in the exponential phase of growth. No independent rounded ribosomes are to be seen, but the cytoplasm appears to be arranged in interconnected cords. The cords appear to be built on a framework of nuclear fibrils (*F*) and to extend from the nucleoplasm (*NP*) towards the plasma membrane. At *PM* opaque fibers contact the plasma membrane. *CW*, layers of the sinuous cell wall. × 240,000.

sufficiently loose for electron microscopic analysis; e.g. the cytoplasm, in comparatively thin sections after Ryter-Kellenberger fixation, of *Escherichia coli* B (Fig. 1) and of the wall-less *Proteus* L form L 9, isolated by Dr. E. Klieneberger-Nobel (Figs. 3, 6, 7). Loosely built cytoplasm is further obtained on removal of the cell wall with lysozyme (Figs. 2, 4 of *Bacillus subtilis* protoplasts) or on relaxing its rigidity by treatment with penicillin (*Proteus vulgaris* (10)). With the description of Figs. 1 to 8 presented here, a preliminary account is given of work in which we analyze the fine structural detail of the sectioned cytoplasm (Figs. 1 to 4) in comparison with that of material released by osmotic shock of protoplasts (Figs. 5 and 8) and L forms, particularly after enzymic degradation (Figs. 6 and 7).

This note is presented in order to attract attention to the existence of fibrillar material throughout the protoplasm. We are aware that in its present state our work is more likely to raise questions than to answer them.

Fig. 1 shows that in thin section the logarithmically growing cells of *Escherichia coli* B are comparatively simple in structure. Intracytoplasmic membranes are rare in this Gram-negative organism. As in all other cases illustrated here, the cell in Fig. 1 was fixed in the presence of 0.01 M magnesium acetate, which is considered suitable for the preservation of ribosomes. There are no independent, discrete particles to be seen in any of our electron micrographs. There are indications in Fig. 1 as well as in Figs. 2 and 3 that the cytoplasm is fibrous rather than granular, with the fibers forming a three-dimensional net of more or less beaded cords. One obtains the impression, particularly in the vicinity of the nuclear area (e.g., *F*), that these cords are built on a framework
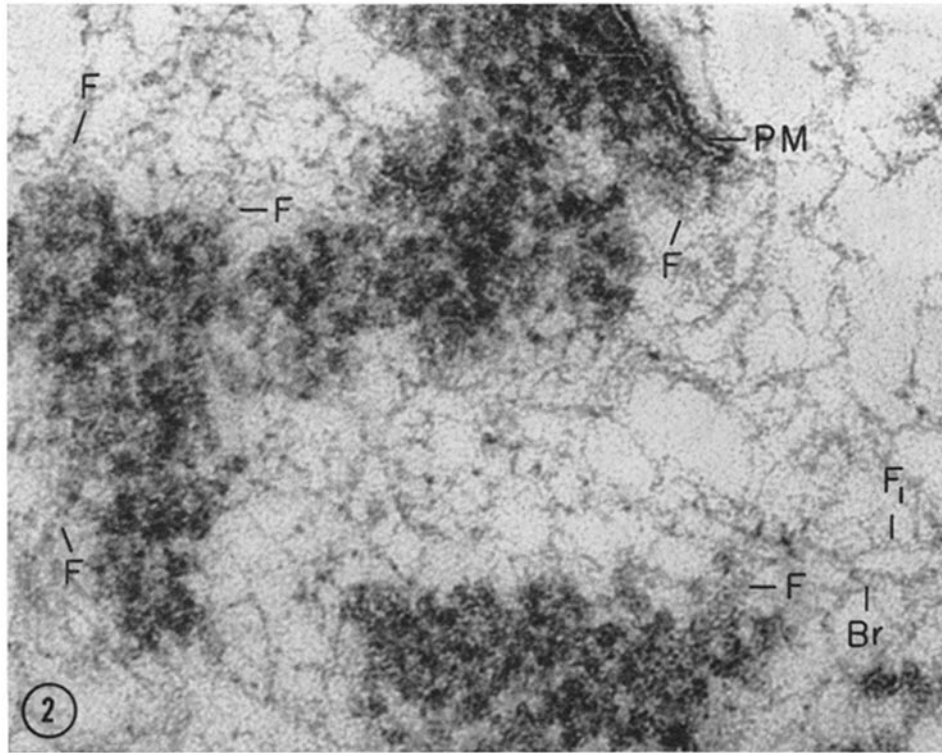
FIGURE 2   From a thin section of a protoplast of *B. subtilis*. The protoplasts were prepared from cells suspended in agar and treated with 0.25% lysozyme at pH 6, in the presence of 0.3 M sucrose. The protoplasts treated in this manner are usually well preserved, but here the plasma membrane (*PM*) is ruptured and fibrillar material from the nucleoplasm extends outwards. The nuclear fibers are arranged in all sorts of aggregates; the larger arrangements (*F*) apparently provide a framework for the cytoplasmic cords. The "beads" on the cytoplasmic cords are about 100 to 175 A. The splitting of a fiber at F_1 seems to result in loops. Note thin strands perhaps interconnecting some fibrils (e.g. *Br*). Such interpretations, however, are submitted with caution in view of the relative thickness of a thin section. × 210,000.

of nuclear fibrils. In the periphery of the cell the cytoplasmic cords contact the plasma membrane (*PM*).

Fig. 2 is of a protoplast of a 4-hour-old cell of *Bacillus subtilis* treated with lysozyme under conditions that minimize swelling and cell rupture. Even so, in this particular protoplast the plasma membrane (*PM*) appears torn, thus releasing the fibrillar material of the nucleoplasm. The nuclear fibrils do not appear as a smooth, single thread, as was observed by Kleinschmidt et al. (11), but rather as a composite system of both single and compound fibrils. Measurements would appear to indicate that most threads are of the order of 20 to 35 A in diameter, whereas very thin ones would

appear to be about 10 A. However, such measurements are, perhaps, of no value in view of the section thickness and the granularity (noise) in the micrograph. At F_1 a fiber can be seen splitting into loops, whereas at *Br* very short fibers appear perpendicular to longer ones; but again these details are so close to the dimensions of the background granularity that their real existence could be questioned. In places in which cytoplasm and nucleoplasm come together, nuclear fibrils (*F*) appear to intermingle with the cytoplasm.

Fig. 3 seems to provide a striking example of the proposed principle of the construction of the cytoplasm on a groundwork of fibrils extending from the nuclear area. In the *Proteus* L form
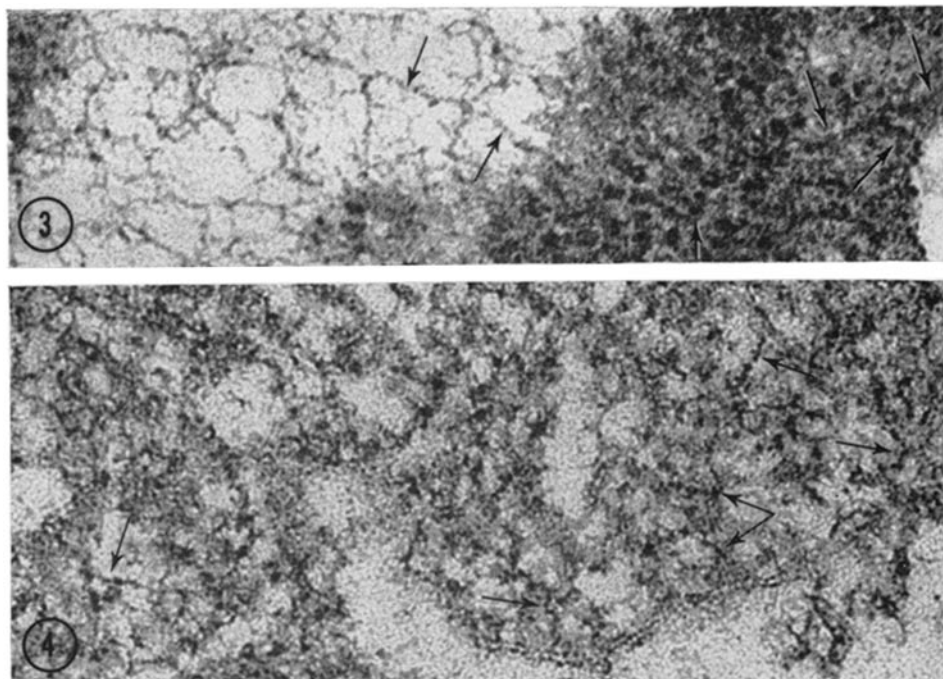
FIGURE 3 From a thin section of a *Proteus* L form. A long fiber from the nucleoplasm enters the cytoplasm and can be followed, by the clustering of the cytoplasmic components, all along its course (arrows) outwards towards the plasma membrane. The more opaque dots in the cytoplasm probably correspond to the ribosomes. × 200,000.

FIGURE 4 From a thin section of a *B. subtilis* protoplast prepared by treatment with lysozyme directly in 0.37 M phosphate buffer, pH 6, in the presence of 0.01 M Mg^{2+} and 0.25 M sucrose. Under these conditions of preparation, the texture of the cytoplasm is loosened and appears to be composed entirely of fibrils of varying diameter, some denser than others (arrows). × 210,000.

illustrated, a long fiber from the nucleoplasm can be traced all the way to the plasma membrane by the arrangement of the cytoplasmic material (*see* arrows).

A general feature of bacterial cytoplasm seems to be that it includes various kinds of fibers, some rather opaque (arrows, Fig. 4), others less so, and yet others occasionally appearing as barely resolvable, tight helices. This feature is illustrated by Fig. 4, of a *B. subtilis* protoplast. The swollen condition of the protoplast allows for greater resolution. The loosening of the texture of the cytoplasm here is, of course, an artifact due to the condition of preparation. But it is an artifact from which it can be learned that, instead of containing discrete ribosomes, the cytoplasm appears to consist basically of various kinds of fibers, the thinnest being, in all such micrographs, close to the limit of resolution.

There is no doubt that, in the electron micrographs of these thin sections, superimposition of structure prevents analysis of the relative positions of the fibrils composing the cytoplasm. Also, the background granularity (noise) seriously hampers the analysis of fibrillar substructure. Of importance, however, is that separate, distinct and uniform particles are not seen to be the main component of this cytoplasm. The beaded cords of electron-opaque matter in Figs. 1 to 3 should correspond to rows of ribosomes. This interpretation conforms, to some extent, to the recently advanced concept of polyribosomes. Yet the present picture may not come really close to what the originators of the term polyribosomes have in mind. It is, therefore, desirable to compare the fine structure of the sections with that of the cytoplasmic material released through osmotic shock and then picked up on carbon-coated grids.
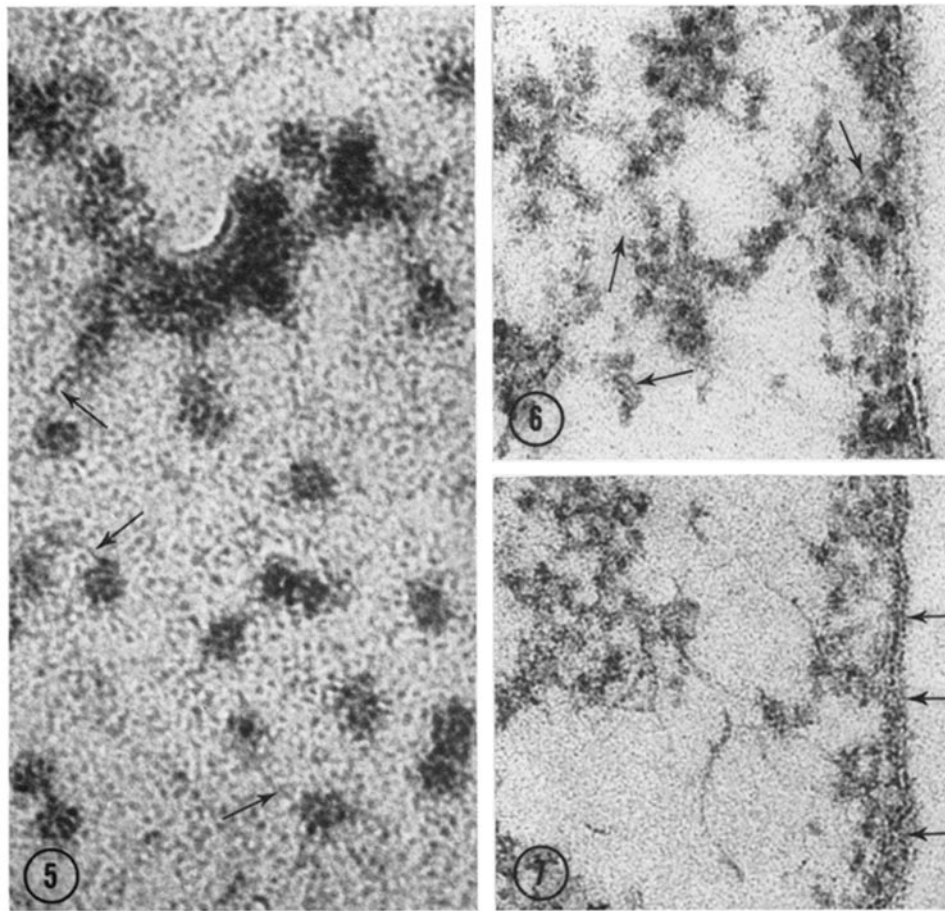
FIGURE 5 Cytoplasmic material from a *B. subtilis* protoplast released by osmotic shock in distilled water with 2% uranyl acetate and 0.01 M Mg^{2+}. Although the protoplast was prepared in the same way as the one in Fig. 4, cytoplasmic particles are now seen which correspond to ribosomes. Monosomes and a few diplosomes can be observed, and in the upper part of the micrograph a cord of cytoplasm. A substructure of electron-opaque fibers is suggested in most cases. From other micrographs, we believe that the substructure extends over large areas of ribosomal material. Delicate fibrils (indicated by arrows) project from these cytoplasmic fragments. × 400,000.

FIGURE 6 Thin section of a *Proteus* L form prefixed in glutaraldehyde in the usual buffer with 0.01 M Mg^{2+}; treated for 1 hr with 0.1% DNase, followed by OsO_4 fixation and uranyl acetate. As in Figs. 1 to 4, this section was stained with lead citrate. In such a preparation, most of the nuclear network disappears but the cytoplasm remains basically fibrillar in structure (arrows), and attached to the plasma membrane. × 230,000.

FIGURE 7 Thin section of a *Proteus* L form prefixed in OsO_4 and treated for 1 hr with 0.1% RNase. A large number of fibrils persist in the nucleoplasm. The remaining cytoplasmic material is arranged on a fibrillar framework in which nuclear fibrils participate. At arrows, fibers contact the plasma membrane. × 230,000.

Fig. 8 shows material from shocked protoplasts released in the presence of cesium and uranyl ion, at pH 5. Again, fibers of various dimensions are seen, the longer ones being branched and associated in some places with a material in which a dense fibrillar substructure can be discerned (as indicated by arrows). Similar material appears dispersed and scattered but also connected to thin strands (F) and strongly resembles the ribosomes of the current literature. These strands connecting the ribosomes to the fibers are often of the order of 450 A, which agrees with the value of 350 to 400 A found by Bladen et al. (1) for strands connecting ribosomes to RNA in their artificial system. However, the ribosomes in Fig. 8 are not attached to a single strand but apparently form part of an interconnected fibrillar system, with several strands joining the particles to long fibers (e.g. F).

Clumps and cords of cytoplasm together with more finely disrupted material (ribosomes, diplosomes, etc.) are likewise obtained when protoplasts are simultaneously shocked and stabilized in distilled water containing ions of uranyl and magnesium (0.01 M) (see Fig. 5). The free particles are variable in size and when "single" we estimate them to be of the order of 100 to 200 A. We believe that in Fig. 5 the granularity in the background is, to a large extent, due to very fine material released from the protoplast. Notwithstanding the limitations imposed by granularities, it is our impression that also in the uranyl-treated material a considerable amount of fine, fibrous material often extends from the ribosomes (see arrows in Fig. 5); some of this material may correspond to the messenger RNA described by others. Obviously, the ribosomes in the uranyl-stained preparations stand out against the background in the micrographs mainly because of the apparent existence within them of electron opaque material. We found many indications, comparable to the situation in the clump or cord of cytoplasm in Fig. 5, that the dense fabric is shared by several ribosomes, and this is a matter requiring further investigation.

Fig. 6 shows a small part of a *Proteus* L form, prefixed in glutaraldehyde, treated with 0.1%

DNase[1] in acetate-Veronal buffer[2] with 0.01 M magnesium acetate for 1 hr at 35°C, then prepared and sectioned as usual. It will be seen that most of the nuclear network disappears whereas the cytoplasmic material remains on a fibrillar framework and attached to the plasma membrane. Likewise, Fig. 7 shows part of a similar organism treated with 0.1% RNase[3] for 1 hr: the cytoplasm partly persists and is built on a fibrillar framework, incorporating nuclear fibers connected to the plasma membrane (see arrows on Fig. 7). In the light microscope the cytoplasm, after the RNase treatment, had lost its reactivity with the Giemsa-stain.

Further experiments are in progress. Our experience so far has convinced us that in bacteria the nuclear fibrillar material is not confined to the region of its highest concentration, but extends from there all through the cell and provides a fibrillar framework for the material of the cytoplasm. By the term "nuclear fibrillar material" it is, of course, not implied that the fibrils everywhere are DNA. We merely want to emphasize the existence of fibrillar continuity between nucleoplasm and cytoplasm. Hence high resolution microscopy of cells fixed in the exponential phase of growth, in the presence of 0.01 M magnesium ions, has engendered serious doubts whether, in bacteria at any rate, the ribonucleoprotein exists as separate ribosomes interconnected only by messenger RNA. The ribonucleoprotein in bacteria appears to be part of an all-pervading fibrous continuum connected to the plasma membrane. On the other hand, it may not yet be justified, on this basis, to conclude that the material obtained in ultracentrifugation studies would represent merely fragments of the ribonucleoprotein continuum. At present, it may be indicated only that there are problems in the visualization of the bacterial ribosomes that are not yet solved. In this context the significance of the various types of threads observed in the thin sections and other preparations merits further investigation.

[1] Worthington's deoxyribonuclease I; 1 × cryst.; pancreatic. This preparation contains some ribonuclease activity.

[2] Acetate-Veronal is often considered not to be a good buffer for glutaraldehyde in electron microscopy. However, when, in the prefixation of these wall-less bacterial cells, glutaraldehyde was used in the usual Ryter-Kellenberger buffer with 0.01 M Mg²⁺, the results closely resembled those obtained when the prefixation was performed with OsO₄.

[3] Sigma's ribonuclease; 5 × cryst.; pancreatic. This preparation is free of protease activity.
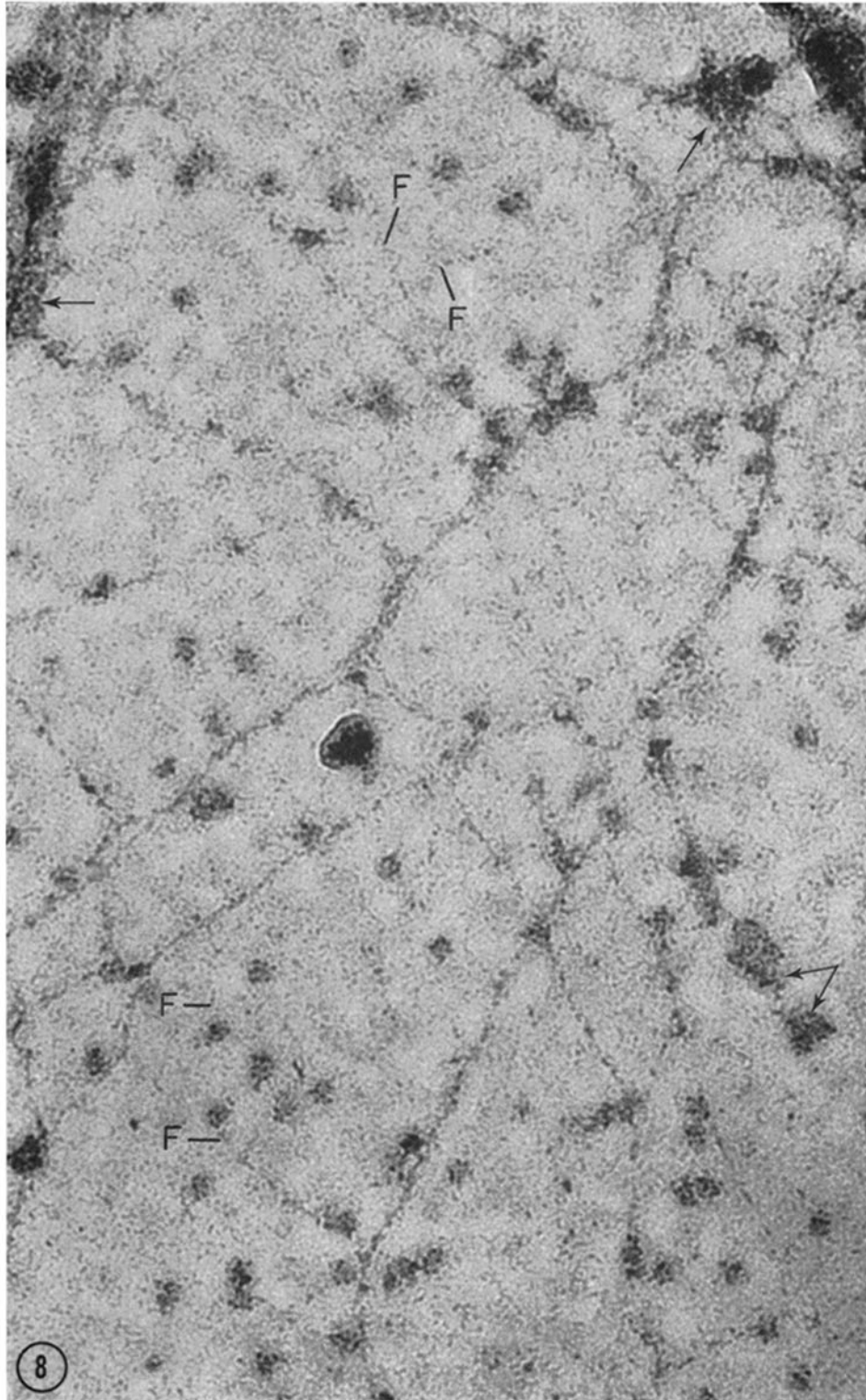
FIGURE 8 Material from a *B. subtilis* protoplast released in distilled water containing 10% cesium chloride and 2% uranyl acetate. The resultant material resembles that in Fig. 5, though the fibers of varying diameter are much better preserved and several are quite long. In some places (arrows), these fibers are associated with matter having a dense fibrillar texture similar to that of the ribosomes in this micrograph. These ribosomes are not entirely free, but frequently appear connected to the longer fibers by very thin strands (*F*). × 210,000.

Note Added in Proof

After this paper had been presented for publication, two articles appeared that must be referred to. In the first article, that of R. G. Hart, *Proc. Nat. Acad. Sc.*, 1965, **53**, 1415, the author describes "surface features" on 50-S ribosomal components from *E. coli* as "arising from a coiled nucleoprotein filament some 35 A in diameter."

The dense fibrillar substructure described by us for the ribosomes released by osmotic shock could correspond with the "coiled nucleoprotein filament."

However, we do not recognize, in our material, the regularity in arrangement similar to that proposed by Hart in his wire model.

The second article, that of D. Schlessinger, V. T. Marchesi, B. C. K. Kwan, *J. Bact.*, 1965, **90**, 465, arrives at a conclusion remarkably close to our concept. The ribosomes of *B. megaterium* are considered as "distributed on a reticular matrix which extends throughout the cytoplasm." In our discussion we have gone, of course, still further by suggesting that the ribosomes themselves would be part of the "reticular matrix."

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