Short papers submitted expressly for this section, reporting original and significant findings of immediate interest and judged to be acceptable without major revision, will be published within approximately three months. See inside back cover for details.

FINE STRUCTURE OBSERVED IN 50S RIBOSOMAL SUBUNITS OF *BACILLUS SUBTILIS*

N. NANNINGA. From the Laboratory of Electron Microscopy, University of Amsterdam, The Netherlands

In the course of a study on the arrangement of the ribonucleoprotein in the bacterial cell (12), structural features were observed in purified 50S particles isolated from *Bacillus subtilis*. A thorough understanding of isolated purified components of the ribonucleoprotein appears to be a prerequisite for an understanding of the organization of the cytoplasm. Although much work is being done on the biochemical properties of the ribosomes, the architecture of these minute organelles is, as yet, little understood. The ribosomes of *B. subtilis*, like those of *E. coli* (21), are composed of 50S and 30S subunits (23). Few interpretations of the fine structure of the purified 50S component of bacterial ribosomes have been published.

From electron micrographs of shadowed preparations, Hall and Slayter (7) interpreted the 50S subunit as being an almost spherical, slightly flattened particle measuring 170 x 140 A. Huxley and Zubay (11) presented similar results after negative staining with phosphotungstic acid. The 50S particles are described as dome-shaped, measuring approximately 160 x 160 x 130 A. Spirin et al. (18) proposed a model for the 50S subunit derived from a heterogeneous mixture of particles obtained from cells treated with chloromycetin (CM particles). These CM particles were found to contain 75% RNA and 25% protein (14), whereas intact 50S and 30S ribosomal subunits would contain 63% RNA and 37% protein (21). The CM particles have sedimentation coefficients of 25S and 18S (17), and are supposed to be the protein-deficient precursors of the 50S and 30S components, respectively (19). The 25S CM particle would consist of five large subunits arranged in a pentagon, and this structure would be basic to the normal 50S particle (18, 13). The 50S subunit is supposed to be constructed from one folded ribonucleoprotein strand (18). In a hypothetical model the 50S particle resembles a pentagonal prism (18).

Hart (8, 9), using a highly purified frozen-dried 50S preparation, arrived at a demonstration of surface features by means of the shadow-casting technique. These features are interpreted as arising from an approximately 35-A-thick, coiled ribonucleoprotein filament similar to that visualized by Spirin et al. (18). Hart's point of view is illustrated by a tentative wire model showing a rounded particle (9) instead of Spirin's pentagonal prism.

To the best of our knowledge, no fresh proposals for the "compact" ribosomal fine structure have been published since the suggestions made by Hart. The term "compact" has been used (18) to stress the difference between normal isolated ribosomes and their so called unfolded forms (18, 22, 5, 6).

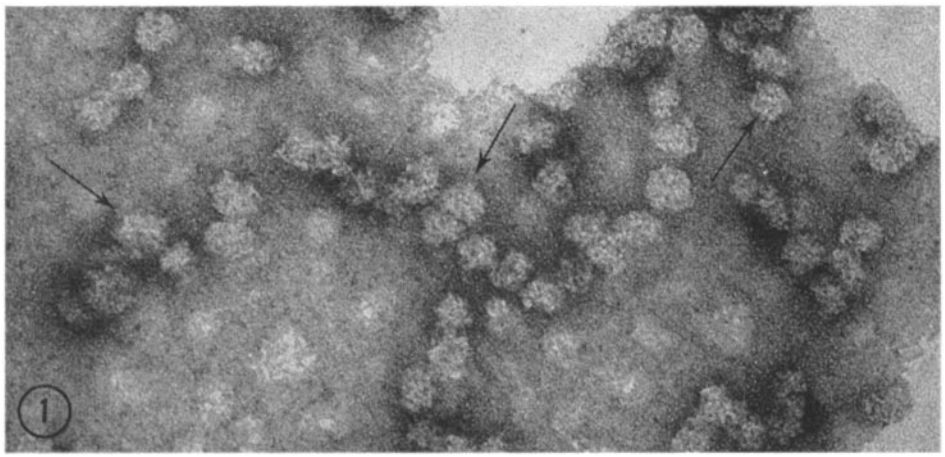


FIGURE 1 A 50S preparation negatively stained with uranyl acetate. Some particles are seen with a pentagonal outline (arrows). Aggregation here and there might be the result of the drying on the grid. × 320,000.

The scanty literature referred to above deals exclusively with the ribosomal particles of *E. coli*. Even less is known of the fine structure of the ribosomes of *Bacillus* species. It, therefore, seems useful to present a few preliminary observations on fine-structural detail observed in purified, compact 50S subunits of the ribosomes of *B. subtilis*.

MATERIALS AND METHODS

Culture and Media

B. subtilis, strain Marburg, was grown aerobically on a shaker in heart infusion (Difco, Detroit) or brain-heart broth (Difco) at 30°C. Cells were harvested during exponential growth, and washed once with 0.1 mM magnesium acetate and 5 mM Tris-HCl pH 7.2 (Tris buffer). Washed cells were kept frozen until used.

Preparation of Crude Extract

A portion of about 7 g of stored frozen cells was thawed and treated three times with liquid nitrogen. All subsequent isolation steps were carried out near 4°C. To the thawed sample were added 3 ml of Tris buffer, 5 g alumina,[1] and 250 μg deoxyribonuclease (ribonuclease free, code DPFF, from Worthington Corp., Freehold, N.J.). After being ground, the cell debris were removed by means of two 10-min centrifugations at 9,000 and 77,000 *g* in an MSE 40 preparative ultracentrifuge. The crude extract was used immediately or stored frozen.

[1] Aluminium oxide (ALCOA-A 301).

Isolation of the 50S Ribosomal Subunit

Portions of 0.5–1 ml crude extract were placed on 16-ml of linear sucrose gradient (5–20%) prepared in Tris buffer. The tubes were centrifuged at 4°C for 4 hr at 100,000 *g* in a MSE50 preparative ultracentrifuge with a No. 2418 swinging-bucket rotor. After centrifugation, 29 fractions consisting of 20 drops each were collected. Four drops of each fraction were diluted with 2 ml of distilled water, and the optical density read at 260 mμ in a Zeiss PMQ II spectrophotometer. From the absorption profile, the 50S subunit was localized and separated from the remaining fractions. The suspension was dialyzed overnight against 500 volumes of Tris buffer. As judged from electron micrographs, this preparation was not yet homogeneous. The 50S suspension was, therefore, centrifuged a second time in a sucrose gradient, and again dialyzed overnight.

In an effort to demonstrate more of the ribosomal substructure, some preparations were treated with cesium chloride, high concentrations of which are known to split part of the protein from ribosomes (4, 16, 10). Purified particles were kept for 52–54 hr in 10% (w/v) CsCl (Ultraspur, from E. Merck A. G., Darmstadt, West Germany) in the presence of 0.025 M magnesium acetate.

Electron Microscopy

For electron microscopy, the particles were fixed for a few hours with 1 volume of 3.5% formaldehyde in Tris buffer. A drop of the suspension was placed on a carbon-coated copper grid and immediately removed with filter paper. This procedure was repeated with a drop of doubly distilled water. While still moist, the

grid was floated downwards on a drop of 0.5% (w/v) uranyl acetate, pH 4.4, in doubly distilled water containing 0.1 mM magnesium acetate, for about 1 min. The uranyl acetate was sucked off, and the specimen was air dried before being examined in the electron microscope. Some of the fields on the grid were found to be negatively stained, others positively. For shadow-casting, a fixed, freshly air-dried preparation was contrasted with carbon-platinum at an angle of 5:1. Micrographs were taken with a Philips EM 200 equipped with a cooling device. The calibration was checked with a grating replica (58,400 lines/inch).

OBSERVATIONS AND COMMENTS

The preparation of the 50S particles obtained by the method described was seen in the electron microscope to be fairly homogeneous. The particles appear to be of more or less equal size (Fig. 1),

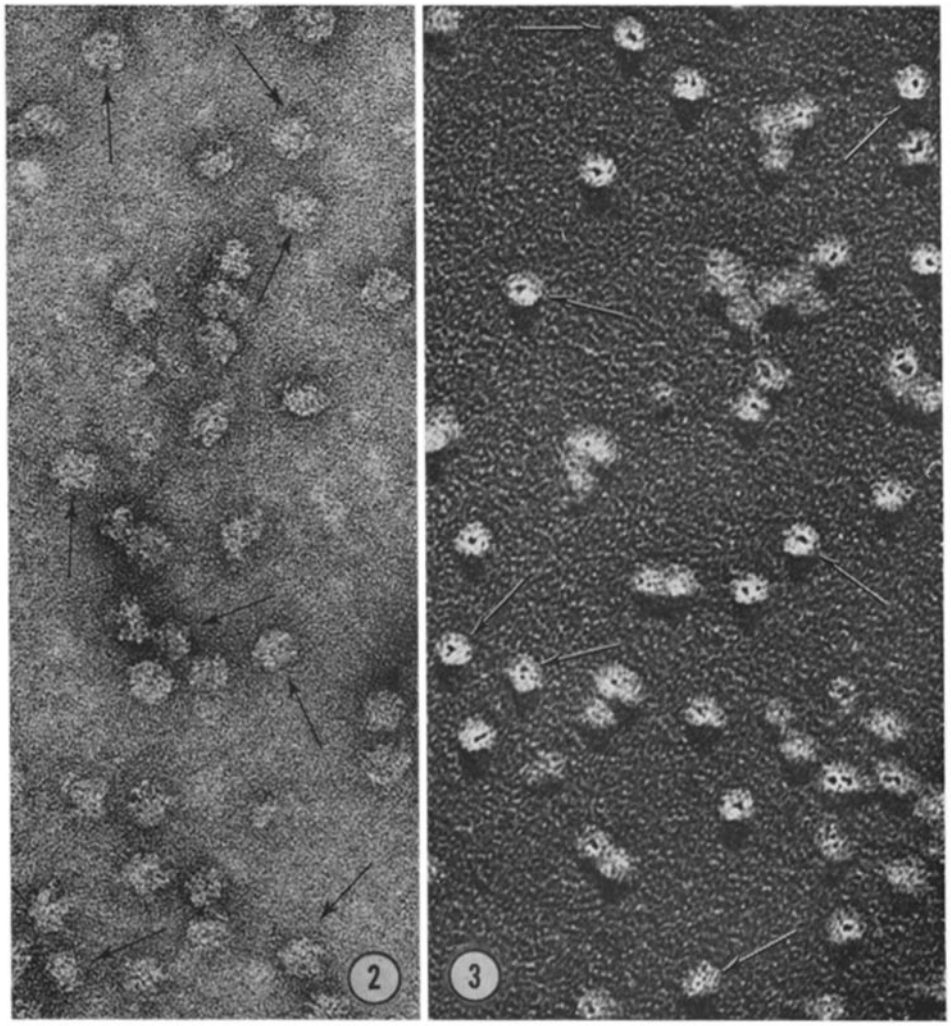


FIGURE 2 A 50S preparation treated for 53 hr with cesium chloride in the presence of 0.025 M magnesium acetate. No obvious effect of the CsCl can be noticed. Many particles display a pentagonal profile (arrows). Particles negatively stained with uranyl acetate. × 320,000.

FIGURE 3 Carbon-platinum–shadowed 50S subunits. Several particles show a pentagonal outline (arrows); many have holes in the center of the pentagon. This is presumably caused by the activation by CsCl of a RNase present in the preparation. The particles appear to be slightly relaxed. × 190,000.

though occasionally some are found aggregated presumably as a result of air drying. Figs. 2, 3, and 5–7 illustrate specimens treated with cesium chloride. The effect of the CsCl in these preparations is somewhat ambiguous. Some of the fields on the grids showed no changes whatever (cf. Fig. 1 and Fig. 2). In these electron micrographs, a certain number of the 50S particles are seen to possess a pentagonal outline (arrows).

In Fig. 3, a carbon-platinum–shadowed, CsCl-treated specimen is shown in which many particles appear somewhat pentangular, and nearly all have holes about 30-A wide. These holes seem to be located in the center of the pentagon. It is, as yet, impossible to decide whether the holes penetrate the whole particle. The shortness of the shadow indicates that the particles have been flattened along the axis normal to the supporting membrane.

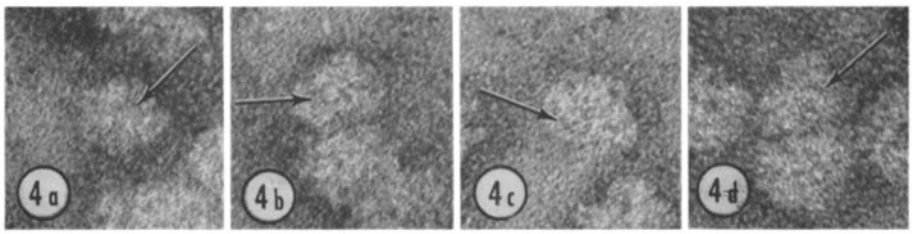


FIGURE 4 *a–d* Some selected 50S subunits negatively stained with uranyl acetate showing a rounded area in the center of the pentagon. × 690,000.

Their width is approximately 250 A, whereas the negatively stained particles as shown in Figs. 1 and 2 were found to measure about 160–180 A across, a value which corresponds fairly well with values for *E. coli* (7, 11). The structure of the particles in Fig. 3 has perhaps been relaxed by a CsCl-activated ribosomal ribonuclease which may have caused some breaks in the RNA chain. It is, therefore, possible that the holes are not only the result of disappearance of material (RNA or protein). Similar holes were observed by Bladen et al. (2) and by Benedetti et al. (1) after treating ribosomes with pancreatic RNase. In our material, these holes have so far been seen only in shadowed specimens. Of particular interest is, however, the appearance of a small rounded area in electron micrographs of the untreated (i.e. normal) negatively stained 50S particles (Fig. 4 *a–d*). On close examination, it is frequently possible to discern such a rounded structure (40–60 A) where one would expect the site of the holes in the particles of Fig. 3.

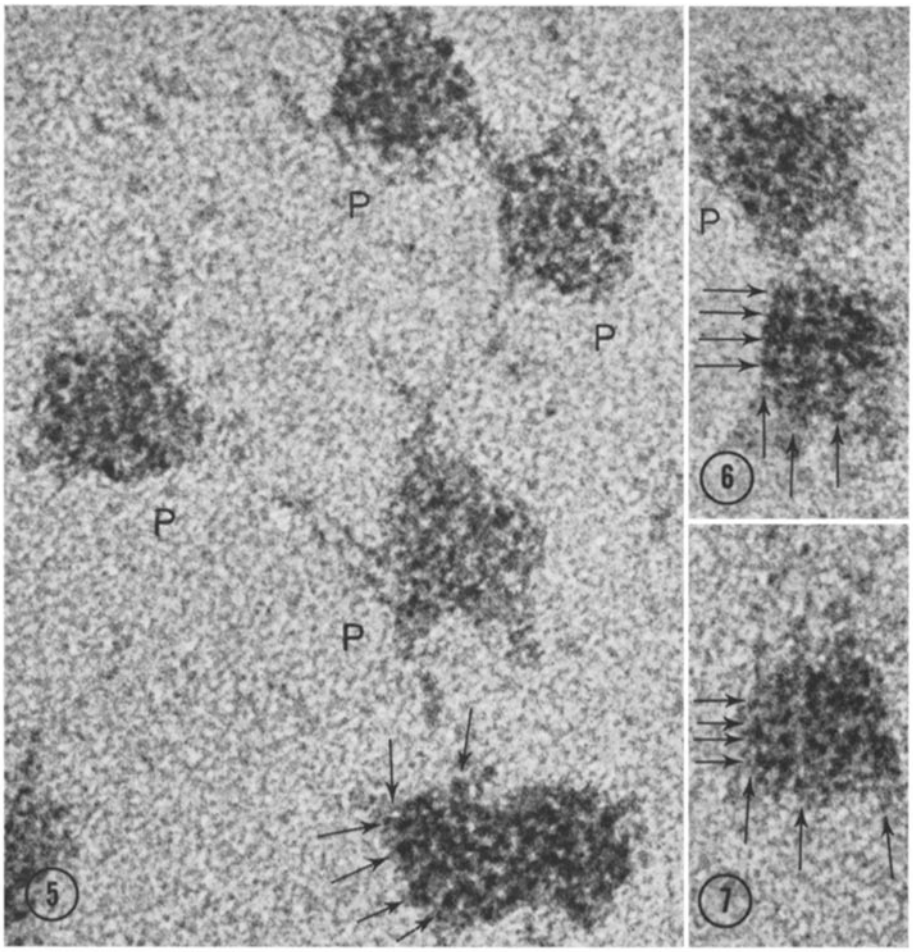
Spirin's deduction of the pentagonal outline of the 50S and the 25S CM particles is based on specimens showing a mixture of particles of different sizes and shapes. The particles in our preparations are of uniform size, but not all of them are five-cornered (Figs. 1–6). This is supposedly caused by the position of the particles on the grid, and perhaps the drying of these fragile structures during preparation for electron microscopy may also have distorted their shape. A major difference between Spirin's specimens and ours is that those of Spirin et al. (18, 13) contained rectangular structures. In none of the specimens here investigated were particles of this particular shape observed with certainty. The rectangular structures in the CM particle preparation were first described as 19S particles (18), but were later believed to be the side view of the 25S particle (20, footnote p. 181). It might be important in this connection that Bradley (3) stressed the close similarity between the rectangular structures found amongst the CM particles and the isolated 22S protein particles obtained after cultivating *Pseudomonas aeruginosa* in the presence of mitomycin C.

From areas stained positively with uranyl acetate were obtained some electron micrographs in which, apart from the pentagonal outline, substructures in the ribosomal particles are shown unusually clearly (Figs. 5–7). Huxley and Zubay (11), positively contrasting with uranyl acetate, described a certain fenestrated or honeycomb fine structure in the ribosome which must be the result of preferential staining of the nucleic acid. Likewise, Figs. 5–7 suggest a "network" of threads in the particles distinguishable by its electron opacity; this may be explained as the configuration of the

ribosomal RNA in projection. The particles indicated by arrows have an uneven density distribution of dense "spots" which show some alignment. In Figs. 6–7, a "vertical" alignment of dense spots (arrows), and a "horizontal" striation perpendicular to it (arrows), can be tentatively made out. The "horizontal" striations, although less straight, are shown even more clearly in the particle, as indicated by arrows in Fig. 5. This particle appears to be almost double, but since its striation is continuous, it most probably represents a single, opened up, ribosomal subunit. The presence of a structure perpendicular to this striation (vertical arrows), though perhaps discernible, is less obvious in this presumably extended particle than in Figs. 6–7.

From several of the ribosomal subunits in Fig. 5, threads seem to protrude, but their course cannot be traced because of the superimposed background granularity which is inevitable at this high magnification (× 1,320,000).

The particles marked *P* in Figs. 5–6 show the pentagonal outline, but in those indicated by arrows in Figs. 6–7, this is lacking. We wonder whether this is because the particles are here lying on their sides. For the time being, it is impossible to conceive how the gridlike pattern of aligned electron-opaque spots is arranged three-dimen-



FIGURES 5–7 CsCl-treated 50S subunits positively stained with uranyl acetate. The particles designated *P* have a pentagonal outline and reveal in the plane of the micrograph a network of fine electron-opaque threads presumably representing mainly RNA. Others show a more or less regular grid pattern as indicated by the arrows. Possibly the latter are particles lying on their sides. × 1,320,000.

sionally inside the ribosomal particle. No evidence could as yet be found in our preparation to substantiate that basically the particles are faceted pentagonal prisms (18, 13, 20).

The distinct pattern of threads constituted by the electron-opaque spots in the 50S particles confirms and extends the observations made by Huxley and Zubay (11) on positively stained ribosomes. Electron-opaque threads in the ribonucleoprotein component of the cytoplasm in thin sections of bacteria have been described by van Iterson (12). Hart's model differs from the present observations in that it lacks the pentagonal face (9). It is not possible to judge whether the course of the bent wire corresponds with the dense threads in the high resolution micrographs here presented. A striking point of convergence between Hart's work and our study is the finding of the "eye-like" region (Fig. 4 *a–d*).

Langridge (15), basing his interpretation on X-ray diffraction patterns of whole ribosomes, suggested for ribosomes of various organisms, including *E. coli*, "an array of four or five parallel RNA double helices, 45 to 50 A apart." It is tempting to speculate that perhaps the "horizontal" striation shown in Figs. 5–7 represents the projection of these parallel RNA double helices.

Our observations seem to warrant the following conclusion: the 50S subunit of the *B. subtilis* ribosome, purified and isolated for electron microscopy, appears as a compact particle about 160–180 A in diameter. In projection in electron micrographs, the particles appear to possess one pentagonal face. Presumably oriented centrally in respect to this face is a rounded area 40–60 A in diameter. The ribonucleoprotein in the particle is organized in a regular pattern, the interpretation of the spatial orientation of which has to remain as yet fragmentary. Projected in a plane presumably perpendicular to the pentagonal face, this pattern appears as a number of lines perpendicular to each other. Further work is required to find out how the three-dimensional organization of the ribonucleoprotein strand can be made to account for both the "eye-like" central region and the pentagonal face of the particle.

The author wishes to express his sincere gratitude to Dr. W. van Iterson for her guidance and encouragement throughout this work, and for her help in preparing the manuscript. Thanks are also due to Dr. D. Stegwee for the generous facilities granted at the Laboratory of Plant Physiology. The skilled assistance of Miss D. Konter, Mr. P. J. Barends, and Mrs. J. Raphael-Snijer is gratefully acknowledged.

Received for publication 2 February 1967.

REFERENCES

1. Benedetti, E. L., W. S. Bont, and H. B. Bloemendal. 1966. *Lab. Invest.* **15**:196.
2. Bladen, H. A., R. Byrne, J. G. Levin, and M. W. Nirenberg. 1965. *J. Mol. Biol.* **11**:78.
3. Bradley, D. E. 1966. Sixth International Congress for Electron Microscopy, Kyoto. Maruzen Co., Ltd. **2**:115–116.
4. Brenner, S., F. Jacob, and M. Meselson. 1961. *Nature.* **190**:576.
5. Gavrilova, L. P., D. A. Ivanov, and A. S. Spirin. 1966. *J. Mol. Biol.* **16**:473.
6. Gesteland, R. F. 1966. *J. Mol. Biol.* **18**:356.
7. Hall, C. E., and H. S. Slayter. 1959. *J. Mol. Biol.* **1**:329.
8. Hart, R. G. 1962. *Biochim. Biophys. Acta.* **60**: 629.
9. Hart. R. G. 1965. *Proc. Natl. Acad. Sci.* **53**:1415.
10. Hosokawa, K., R. K. Fujimura, and M. Nomura. 1966. *Proc. Natl. Acad. Sci.* **55**:198.
11. Huxley, H. E., and G. Zubay. 1960. *J. Mol. Biol.* **2**:10.
12. Iterson, W. van. 1966. *J. Cell Biol.* **28**:563.
13. Kisselev, N. A., and A. S. Spirin. 1964. Proceedings of the Third European Conference on Electron Microscopy. Publishing House of the Czechoslovak Academy of Sciences, Prague. **B**:39–40.
14. Kurland, C. G., M. Nomura, and J. D. Watson. 1962. *J. Mol. Biol.* **4**:388.
15. Langridge, R. 1963. *Science.* **140**:1000.
16. Meselson, M., M. Nomura, S. Brenner, and D. Schlessinger. 1964. *J. Mol. Biol.* **9**:696.
17. Nomura, M., and J. D. Watson. 1959. *J. Mol. Biol.* **1**:204.
18. Spirin, A. S., N. A. Kisselev, R. S., Shakulov, and A. A. Bogdanov. 1963. *Biokhimiya.* **28**: 920.
19. Spirin, A. S. 1963. *Cold Spring Harbor Symp. Quant. Biol.* **28**:267.
20. Spirin, A. S. 1964. Macromolecular Structure of Ribonucleic Acids. Reinhold Publishing Co., New York.
21. Tissieres, A., J. D. Watson, D. Schlessinger, and B. R. Hollingworth. 1959. *J. Mol. Biol.* **1**:221.
22. Weller, D. L., and J. Horowitz. 1964. *Biochim. Biophys. Acta.* **87**:361.
23. Woese, C. R. 1961. *J. Bacteriol.* **82**:695.