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Two-dimensional crystalline sheets of *Bacillus stearothermophilus* 50S ribosomal particles

T. Arad, K. Leonard, H.G. Wittmann[1]* and A. Yonath[2]

European Molecular Biology Laboratory, D-6900 Heidelberg, [1]Max-Planck-Institut für Molekulare Genetik, D-1000 Berlin-Dahlem, FRG, and [2]Weizmann Institute for Science, Rehovot, Israel

*To whom reprint requests should be sent

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Two-dimensional crystalline sheets of the large ribosomal subunit from *Bacillus stearothermophilus* have been obtained using a slightly modified procedure to that for growing three-dimensional crystals of the same material. The crystalline subunits are packed within monolayers in a relatively small unit cell, the dimensions of which are closely related to those observed for two forms of the three-dimensional crystals. The packing symmetry is p121, and the optical diffraction patterns of micrographs of negatively stained crystals extend to ~3.0 nm.

Key words: Bacillus stearothermophilus/50S ribosomal subunit/crystallography

Introduction

Ribosomes are complexes of proteins and RNA chains providing the site for protein biosynthesis in every organism. The biological, chemical, physical and genetic properties of prokaryotic ribosomes have been studied extensively (Chambliss *et al.*, 1980; Liljas, 1982; Wittmann, 1982, 1983). Because of their size and complexity, ribosomes are also ideally suited for study by electron microscopy. Extensive structural work on ribosomal subunits of *Escherichia coli* has been carried out by direct visual interpretation of electron microscope images. This has led in the past to different models for the 30S and 50S subunits and for the way in which they form the 70S ribosome. A more objective structural model for the ribosome and its subunits can be obtained by carrying out three-dimensional image reconstruction from electron micrographs.

Working with negatively-stained ribosome suspensions it is possible to obtain a three-dimensional model from full tilt image series of single particles (Knauer *et al.*, 1983; Oertl *et al.*, 1983), by correspondence analysis of different particle orientations of untilted micrographs (Frank *et al.*, 1982; Frank and van Heel, 1982; van Heel, 1981) or from a combination of images of single particles using a small number of tilts (Provencher and Vogel, 1982).

The use of periodic arrays or crystals of ribosomes would, however, have advantages in reducing the damaging effects of specimen preparation and radiation. It would also enable the use of the well established diffraction techniques which are based on Fourier averaging and tilt reconstruction to be carried out. These periodic arrays should preferably be two-dimensional (i.e., single layer) crystals.

Such two-dimensional crystals are formed *in vivo*, under special conditions, for the less well biochemically defined eukaryotic ribosomes (Byers, 1967; Kress *et al.*, 1971; Taddei, 1972; O'Brien *et al.*, 1980; Barbieri, 1982; Kühlbrandt and Unwin, 1982). They have, however, so far proved difficult to form *in vitro* for the well-defined prokaryotic ribosomes or their subunits. Tubes and single sheets for *E. coli* 50S particles have been previously obtained, for which the unit cell consists of a tetramer of 50S subunits (Clark *et al.*, 1982). We wish to report here the preparation of a new two-dimensional crystal form of 50S particles for *Bacillus stearothermophilus* which has two 50S particles per unit cell and which is suitable for tilt reconstruction by the Fourier method.

Results and Discussion

Three-dimensional crystals of *B. stearothermophilus* 50S particles have previously been obtained by vapour diffusion using various organic solvents (Yonath *et al.*, 1980, 1983). These are too thick to be studied directly by electron microscopy, and we have attempted to obtain three-dimensional information for the individual 50S particles by image analysis of thin sections of the crystals (Leonard *et al.*, 1982). This work is, however, somewhat limited by the lower resolution of the sectioned material, by the sectioning direction being not absolutely defined and by the difficulty of interpreting positively stained images.

Two-dimensional crystals which can be visualised directly in the electron microscope have now been obtained by essentially the same crystallisation method, but using: (a) an active, pure preparation of the 50S subunit that had failed to produce three-dimensional crystals detectable by light microscopy, and (b) a variation of the relative concentrations of the materials in the crystallising medium (i.e., lowering the subunit concentration and increasing the Mg^{2+} concentration). Crystals start forming reproducibly after 4 days, and reach their optimum size after 9 – 14 days. The maximum size of the crystals which we have observed is ~1 μm long in the unit cell *a* direction and 0.3 μm long in the *b* direction.

The crystals are suitable for study by negative staining and thus to yield a 'conventional' model for the 50S particle. Optical diffraction patterns for micrographs of crystals negatively stained with uranyl acetate (Figure 1) gave a maximum resolution of ~0.3 nm (Figure 1c). The unit cell dimensions are 14.5 ± 0.3 nm x 31.1 ± 1.4 nm and the unit cell angle is 89° ± 2.6°. As shown in Table I, these dimensions agree well with those previously obtained for two forms of the three-dimensional crystals of the same 50S particles, for which data have been obtained both by electron microscopy, using positively stained thin sections (Yonath *et al.*, 1983), and by detection of periodic spacings on X-ray screenless precession patterns (Yonath *et al.*, 1982). The small variations in cell constants between the different crystal forms may arise from the differences in the crystallization conditions as well as from the treatment needed for the preparation for electron microscopy.

Optical diffraction patterns extend to about 10th order in the b^* direction (3 nm), and they appear to show systematic

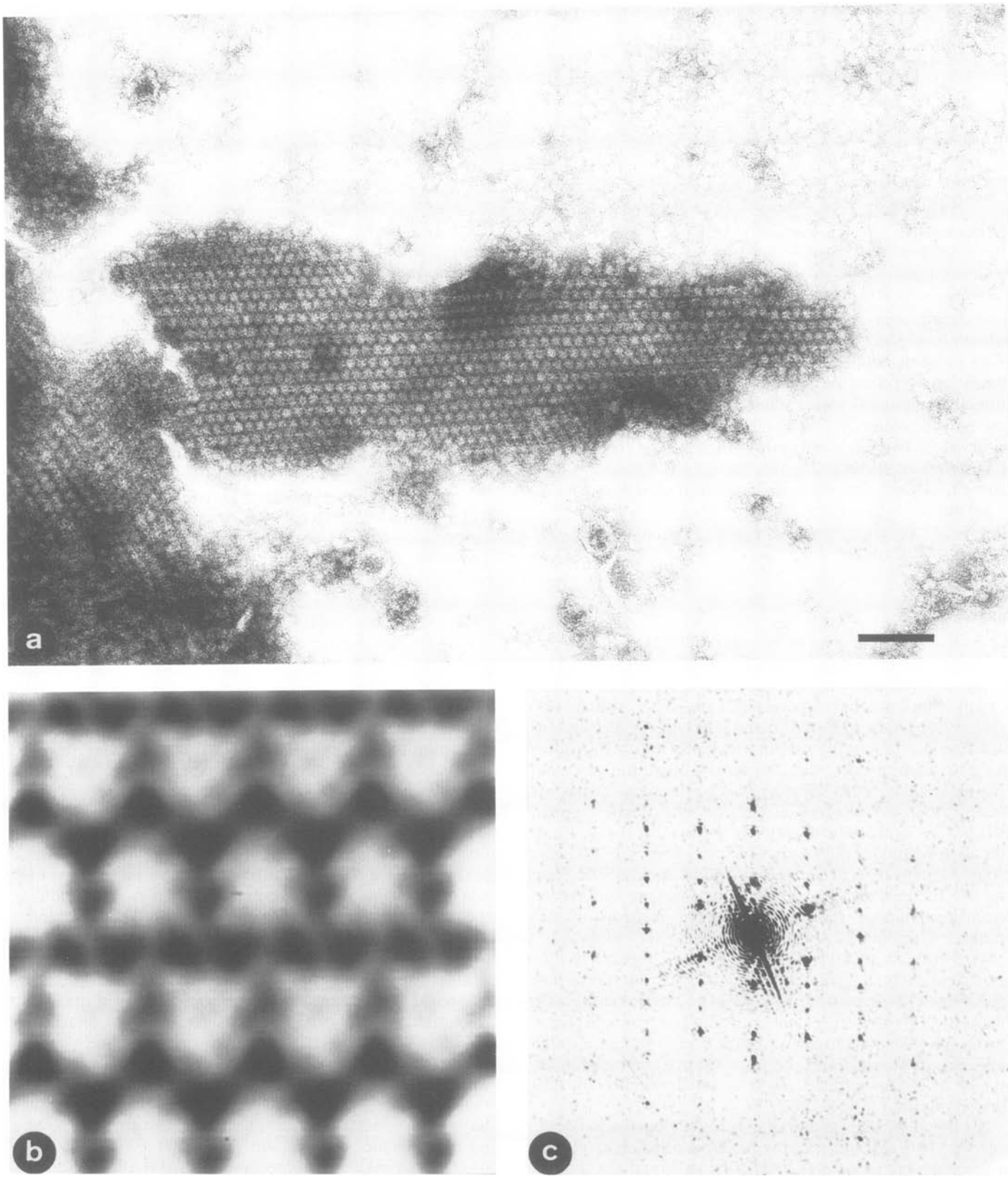

Fig. 1. **(a)** Electron micrograph of a negatively stained crystalline sheet of 50S subunits. Bar length is 100 nm. **(b)** Noise filtered image of the electron micrograph shown in **a**. **(c)** The corresponding optical diffraction pattern.

absences along the a^* direction but not the b^* direction (the reflection 0, 5 is always strong). This would suggest that the crystals have plane group symmetry pg in projection, with a glide plane along the a direction.

Figure 1b, which is a computer noise filtered and averaged image of the crystal in Figure 1a, shows the outline of the par-

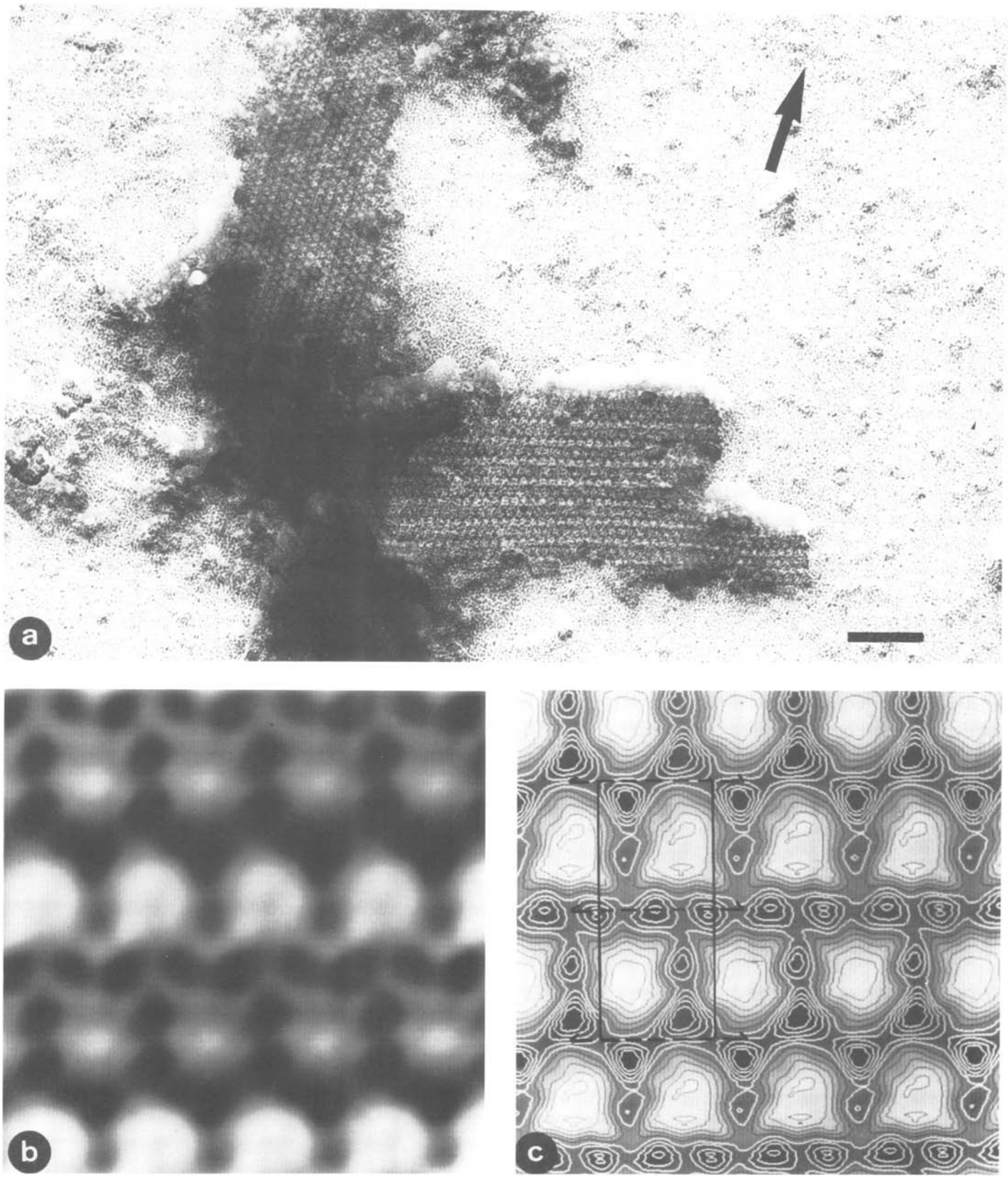

Fig. 2. (a) Electron micrograph of a Pt-Pd shadowed crystalline sheet. Bar length is 100 nm. The arrow indicates the direction of metal deposition during shadowing. **(b)** Noise filtered image of **a**. **(c)** Contoured density for the filtered image of **a**. The rectangular unit cell (14.5 nm x 31.1 nm) and the screw axes for plane group p121 have been drawn in.

ticle and also indicates the packing of the subunits within the unit cell. The particles are arranged in alternating rows which show up clearly, both in the original pattern and in its filtered image. However, it is very difficult in this case to distinguish in projection between a dyad axis in the plane of the crystal (i.e., plane group p2) and a screw axis along the *a* direction of

Table I. Cell parameters of two three-dimensional crystal forms from *B. stearothermophilus* 50S ribosomal particles

Method	Crystal form	Cell parameters		Crystal conditions
		a,b (nm)	γ	
Electron microscopy of thin sections[a]	# 1	13.0 x 25.6	96°	pH 7.0; 10% ethandiol
	# 2	15.6 x 28.8	97°	pH 6.6; 3% ethylhexandiol
X-ray precession pattern[b]		15.4 x 26.1[c]	90°	pH 8.7; 30% methanol

[a]For references see: Yonath *et al.* (1983).
[b]For references see: Yonath *et al.* (1982).
[c]Detected periodic spacings.

the unit cell (i.e., plane group $p12_1$). Results of low-angle unidirectional shadowing seem, however, to differentiate quite clearly between the two possibilities. In Figure 2a it can be seen that if a crystal is shadowed at a fixed low angle approximately along the *b* direction, there is a clear alternation in shadow for adjacent rows parallel to *a*. This is not visible when the shadowing is performed along the *a* direction and can be explained by a variation in height of alternate crystal rows parallel to *a*. This would arise if there were a packing in which alternate 50S particles are oriented up-and-down across the crystal layer, i.e., with a screw axis along *a*. The thickness of the sheets has also been determined directly from the shadowed images, and found to be ~15 nm which is consistent with the presence of a single layer of ribosomes.

To characterize the factors that are responsible for the production of two-dimensional crystals, we have also investigated in more detail the formation of three-dimensional crystals as a function of the Mg^{2+} concentration.

50S preparations which at low Mg^{2+} concentration produce normal three-dimensional crystals were tested for crystal growth over a range of Mg^{2+} concentrations. It was found that when the Mg^{2+} concentration in the crystallization medium exceeds 50 mM, in most cases no three-dimensional crystals could be detected by light microscopy. In some experiments small three-dimensional crystals were produced, but these dissolved within 1–2 days. A hypothesis to explain these results is that the inter-particle contacts which are created in the two-dimensional plane are different from those between planes in the three-dimensional crystal. The latter are subjected to a strong competition with Mg^{2+} ions whereas the intra-plane contacts are either indifferent to, or even enhanced by, the presence of Mg^{2+}. The chemical properties of the two types of contact are still unknown; the competition with Mg^{2+} ions may be very specific, but also may only be an indication for weaker interactions.

To check whether the biological activity of the 50S particles has been impaired during the period of crystallization, a sample of 180 drops was kept for 3 weeks under the crystallization conditions, and another sample was stored for the same time and the same temperature but in the absence of ethanol. As shown by the poly(U) test, the biological activities of both samples were approximately the same, and identical to that of a control sample which was frozen for the 3 weeks. Furthermore, all three samples gave sharp and symmetrical 50S peaks on sucrose gradients.

Although these results reveal that the 50S particles apparently remain intact and biologically active under the crystallization conditions, a note of caution should be added. Since only a small portion of the particles gave crystalline sheets and since the sheets cannot be separated from the majority of the non-crysallized material it is not possible to determine whether or not the biological activity of the crystallized 50S particles has remained to the same extent as that of the non-crystallized ones. A partial loss of activity or the removal of one (or a few) protein(s) from those 50S particles which form the crystalline sheets would be undectable due to the small portion of crystalline sheets and the experimental errors of the applied methods.

Conclusions

The procedure described is a reproducible method of preparing *in vitro* two-dimensional crystals of *B. stearothermophilus* 50S ribosomal subunits which are suitable for direct study by electron microscopy. The optical diffraction patterns of these crystals extend to 3.0 nm and show a regular, compact packing mode with relatively small unit cell (14.5 nm x 31.0 nm). The crystals belong to the two-sided plane group p121 with a screw axis along the unit cell *a* direction. This conclusion is supported by (i) the systematic absences of diffraction spots along the a^* direction (2 h + 1 are missing), (ii) the angle γ being close to 90° and (iii) the alternating image density after shadowing. We are at present using the crystals to carry out a tilt reconstruction which should give a three-dimensional model for the large ribosomal subunit. Use of contrast matching stain (Kühlbrandt and Unwin, 1982) may also enable the position of the RNA to be established. If similar crystals can be obtained for the 70S particle, it should be possible to establish more exactly the way in which the two subunits make up the whole ribosome.

Materials and methods

Ribosomes were prepared as described previously (Yonath *et al.*, 1980). For crystallization, the 50S subunits were diluted in H-I(m) buffer (10 mM HEPES buffer, pH 7.8, containing 60 mM NH_4Cl, 10–90 mM $MgCl_2$ and 6 mM 2-mercaptoethanol) to a final concentration of 2–3 mg/ml and centrifuged for 30 s at 10 000 r.p.m. to remove large aggregated material. This solution was then adjusted to pH 6.0 with 50 mM MES buffer, immediately before setting up for crystallisation. Then 9 μl drops were placed on a siliconised glass plate which was inverted over a glass Petri dish containing a total of 10 ml of 10% ethanol in H-I(m) buffer and 0.5 M NaCl (Yonath *et al.*, 1982). For some crystallisation experiment 1% ethanol was added directly to the ribosome solution.

After 4–14 days at 4°C, crystals were prepared for electron microscopy by re-inverting the glass plate and touching carbon-coated copper grids to the surface of the drops. These grids were then washed with H-I(m) buffer and stained with 1% uranyl acetate.

Micrographs were taken in a Philips 400T electron microscope at 80 kV. Optical diffraction patterns and filtered images were made as described previously (Leonard *et al.*, 1982). Shadowing was carried out at a fixed angle of 20° using Pt/Pd wire in an Edwards evaporator at 10^{-5} Torr and room temperature.

The biochemical methods used in this study were the same as previously described (Wittmann *et al.*, 1983).

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