DIFFRACTION STUDIES ON CRYSTALS OF RIBOSOMAL PARTICLES

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INTRODUCTION

One of the most fascinating biochemical processes is the biosynthesis of protein molecules. This intricate and accurate process takes place in all organisms on their ribosomes in a similar manner. It involves specific interactions of the ribosomes with mRNAs, aminoacyl-tRNAs and a number of proteins such as initiation, elongation and termination factors. During this process the ribosomes dissociate into their two subunits which reassociate after the initiation process. Each of these subunits is a structurally independent distinct assembly of many proteins and several RNA chains.

The structure of ribosomal particles, in particular those from bacterial sources, has been intensively studied during the last two decades by a number of chemical, physical, immunological and genetic methods (for recent reviews see references 1-4). All the ribosomal proteins and RNAs of the Escherichia coli ribosome have been isolated and their primary structures determined. The architecture of the ribosome is currently being investigated by immune electron microscopy, neutron diffraction, chemical cross-linking, and affinity labeling, as well as by fluorescence and other techniques. In spite of the immense progress which has been achieved by these methods, a detailed molecular model for the ribosome has still not been determined.

A few years ago, bacterial ribosomal particles were crystallized in vitro, and diffraction techniques such as X-ray crystallography and three-dimensional image reconstruction from periodically organized specimens have been applied to these crystals. The purpose of this article is to summarize the results of these studies, together with those of diffraction studies on crystalline arrays of ribosomes which occur in vivo in some eukaryotes.

ONE- AND TWO-DIMENSIONALLY ORDERED FORMS OF RIBOSOMES

Under special conditions (such as suboptimal temperatures, oxygen lack, hibernation, etc.) the ribosomes of several eukaryotic species (lizard, chicken, amoeba and human) associate with each other in vivo to form helices and two-dimensionally ordered layers. Some of these ordered

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forms have proved useful for low-resolution structural studies.

a) Helices of chromatoid bodies of the protozoan Entamoeba invadens are probably composed of ribosomes or their precursors[5]. Positively stained sections were used to derive a reconstructed body consisting of three regions of unequal size, named: L and S subunits, and X particle, respectively.

b) Membrane bound double layers of ribosomes from the oocytes of the lizard, Lacerta sicula, are formed during hibernation or after prolonged cold treatment. The ribosomes are organized as tetramers in a P4 lattice of a = 59.5 nm. Comparison of three-dimensionally reconstructed models obtained at 6-7 nm resolution from sheets which were contrasted with gold thioglucose or with glucose, showed that the rRNA chains are concentrated in a central core with few accessible sites on the surface; that the ribosomal proteins are located mainly at the periphery; and that the subunit interface is rich in rRNA. However, the shape of the two subunits of the ribosome could be only partially resolved[6].

c) Tetramers of ribosomes have also been observed as the basic building units of the ordered ribosomal sheets in slowly chilled early chick embryos. These sheets are packed with P422 symmetry. A similar organization could be obtained in vitro. In the latter case two-dimensional arrays of ribosomes with the space group $P42_12$ (a = 59.3 nm) and an internal order of 6 nm were produced. The asymmetric unit in these arrays consists of tetramers of whole ribosomes together with four non-ribosomal proteins. Low angle X-ray diffraction patterns of partially oriented gels of these sheets and three-dimensional reconstructions of negatively stained specimens were used to obtain information about the internal packing within these arrays[7].

d) Hirano bodies, which are stacked sheets of membrane bound ribosomal particles, appear in the brains of senile humans[8]. Spatial Fourier filtering of stained sections revealed that the particles are packed in layers of a rhombic lattice with a = b = 13 nm and $\alpha = 56°$ in which the average interparticle distance is 21.5 nm.

These studies on two-dimensional sheets composed of eukaryotic ribosomes have yielded some useful low resolution information about the modes of packing, the interactions between the particles, the outer contour of the ribosomes and the inner distribution of their components. However, the quality of the results is limited by the very nature of the systems. The organization of the sheets is induced by cellular effects and is expressed in only one or two dimensions. Furthermore, the particles are usually packed in rather large unit cells which only permit relatively low resolution studies. Thus, the only suitable diffraction technique to be applied to these systems is three-dimensional image reconstruction from electron micrographs, a technique which has many merits but also severe limitations.

Ribosomes from bacteria are smaller and have been studied biochemically in much greater detail than those from eukaryotes. They can also be produced in high purity and large quantity. Moreover, they provide a system for crystallization which is independent of in vivo events and environmental influences such as hibernation.

Attempts to produce two-dimensional crystalline sheets from bacterial ribosomes in vitro have been successful in a few cases. Helical arrays of E. coli small ribosomal subunits[9] and crystalline sheets of large ribosomal subunits from the same organism[10] have been reported. The building units of these sheets are tetramers which have lattice parameters of a = b = 33 nm and $\alpha = 123°$, and the space group is most likely $P2_1$.

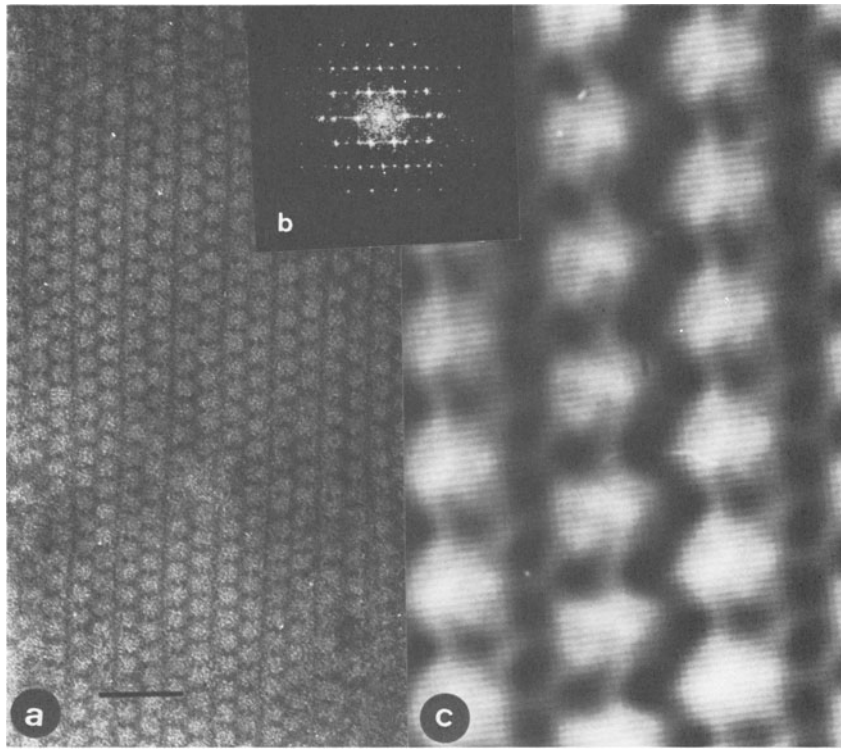

Fig. 1: (a) Electron micrograph of a negatively stained crystalline sheet, (b) its optical diffraction pattern, and (c) its noise filtered image. Bar lengt is 50nm.

Further, two-dimensional crystalline sheets which diffract to the 10th order (3 nm resolution) have been obtained from the large ribosomal subunit of Bacillus stearothermophilus[11] (Fig. 1). In this case two particles related by $P12_1$ symmetry are packed in unit cells with relatively small dimensions (14.5 x 31.1 nm), which agree well with some forms of the three-dimensional crystals of these 50S particles (Table 1). Filtered images of the negatively stained sheets (Fig. 1) show particles with similar dimensions to those revealed by other methods and with the characteristic features that have been detected by electron microscopy of single particles[11].

Table 1: Characterization of crystals of 50S ribosomal particles from B. stearothermophilus using electron microscopy.

Crystal Form	Cell Parameters (nm)	
1*	13.0; 25.4	γ = 95°
2*	15.6; 28.8	γ = 97°
3	26.0; 28.8	γ = 104°
4	40.5; 40.5; 25.6	γ = 120°
5	21.3; 23.5; 31.5	γ = 127°

*May be related to each other.

THREE-DIMENSIONAL CRYSTALS OF RIBOSOMAL PARTICLES

The three-dimensional crystallization of bacterial ribosomes was achieved as a result of a systematic exploration of the parameters that control this process and the development of an experimental procedure for fine control of the content of the crystallization medium[12]. Several three-dimensional crystal forms have reproducibly been obtained from the large ribosomal subunits of B. stearothermophilus[13-15] and from E. coli 70S ribosomes[16].

There is a correlation between crystallizability and biological activity since inactive ribosomal particles could not be crystallized. Moreover, in all cases, the in vitro crystalline material retains its biological activity even for as long as several months, in contrast to the relatively short lifetime of isolated ribosomes in solution. This property is in agreement with the hypothesis that periodic organization of ribosomal particles can occur in vivo when external conditions, e.g. hibernation, demand temporary storage of many ribosomes in a cell.

Electron micrographs of positively stained thin sections of embedded three-dimensional crystals show a regular packing of ribosomal particles with dimensions similar to those previously determined for these particles by various physical techniques such as electron microscopy and small angle X-ray scattering. Several crystal forms were suitable for further structural analysis by three-dimensional image reconstruction.

Image Reconstruction from Positively Stained Thin Sections

Since three-dimensional crystals are too thick to be studied directly by electron microscopy they were embedded and sectioned. The thin sections were positively stained with uranyl acetate which reacts mainly with the nucleic acid moiety of the particles. Cell parameters were determined from the optical diffraction patterns of electron micrographs of these sections[13-16].

Microcrystals of 70S E. coli ribosomes are very well organized, and the particles are packed with hexagonal (P63) symmetry in unit cells of $a = b = 34$ nm, $\gamma = 102°$ and $c = 59$ nm (Fig. 2). Assuming 50-60% hydration, each asymmetric unit consists of one ribosome for which the approximate dimensions (in two directions) could be estimated. Positively stained sections cut parallel to the 6-fold axis in various positions relative to the AB face (Fig. 3) show several views of the distribution of the stain within the particle (Fig. 4).

For the 50S particles from B. stearothermophilus the cell dimensions are in most cases relatively small. For two crystal forms (#1 and #2 in Table 1) they are in good agreement with the periodic spacings determined from their X-ray patterns (see below).

After staining with uranyl acetate the crystal sections were tilted in the electron microscope, and three-dimensional image reconstruction studies were performed for four forms (#1-#4 in Table 1) of 50S crystals of B. stearothermophilus[17]. All four forms show essentially the same stain distribution within the particle. The model obtained from these studies has dimensions similar to those determined by other physical techniques. It consits of two domains of unevenly distributed density. Comparison of this model obtained by staining with uranyl acetate alone with that obtained from sections stained with uranyl acetate together with phosphotungstic acid, shows that the portion of the subunit that interacts with uranyl acetate (presumably the rRNA) is distributed mainly in the core of the particle, whereas the ribosomal components that interact with phosphotungstic acid

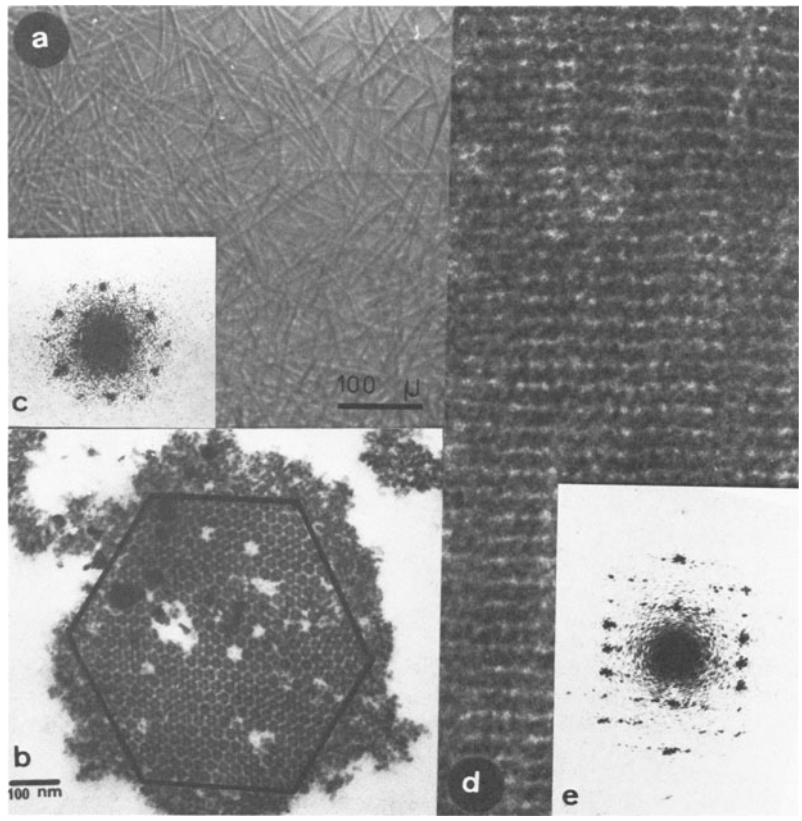

Fig. 2: (a) Crystals of 70S ribosomes from E. coli as seen by light microscopy. Growth conditions: 12% methyl pentane diol at pH 8.7. (b) Electron micrograph of a positively stained thin section across an embedded crystal, the edges of which are depicted. (c) Optical diffraction pattern of (b). (d) Electron micrograph of a section through another crystal of E. coli 70S ribosomes. (e) Optical diffraction pattern of (d).

(presumably the proteins) are located closer to the surface and are involved in interactions between the crystalline particles (unpublished data).

X-Ray Diffraction Studies on Ribosomal Crystals

Preliminary X-ray diffraction patterns have been obtained for crystals of the large ribosomal subunits of B. stearothermophilus from three types of specimens: native single crystals, glutaraldehyde cross-linked single crystals, and large amounts of microcrystals[18]. These studies include:

a) Still and 2° oscillation photographs of chunky medium sized crystals (Fig. 5) and of fragments derived from large (0.9 x 0.25 x 0.15 mm) crystals (Fig. 6) which are relatively stable in the X-ray beam (more than three hours in a synchrotron beam of 5 x 10^8 photons/sec at 28-35 mA). The X-ray patterns include reflections to 1.5-1.8 nm resolution and have periodic spacings of 13.8 ± 0.4 nm and 25.9 ± 0.4 nm (Fig. 7).

b) Screenless 1° precession photographs of a cross-linked fragile needle-like thin crystal (0.15 x 0.05 x 0.05 mm) which diffracts to a resolution of 0.95-1.0 nm with periodic spacings of 15.4 nm and 26.1 nm[18].

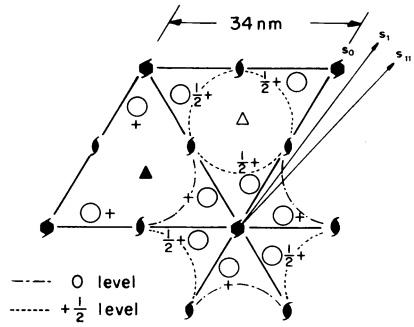

Fig. 3: A schematic diagram of the AB face of crystals of E. coli ribosomes. Suggested outer dimensions of the asymmetric units are drawn. S_0, S_1 and S_{11} define the directions of the sections, the filtered images of which are shown in Fig. 4.

The spacings determined in both of these two approaches mentioned are similar to those obtained by electron microscopy from thin sections of these crystals and from two-dimensional sheets[11]. The small variations between the cell constants measured by electron microscopy and the periodic spacings detected on the X-ray patterns may arise from differences in the crystallization conditions and/or from somewhat different handling during the preparation for electron microscopy.

c) "Powder" diffraction from samples containing large amounts of microcrystals which gave fairly sharp rings both in high and low resolution. Among them are some high angle features with spacings similar to those previously reported for gels or ribosomes and extracted rRNA[19,20]. The patterns are fairly well oriented, and for aligned crystals the average arc length is ± 30°. Such patterns may arise from partial orientation of the nucleic acid component within the particle. The low angle features of these patterns clearly shows two components. One of these arises from the shape of the particles and gives parameters that agree well with those measured previously for E. coli 50S non-crystallized subunits[21]. The other component consists of some crystalline sharp, oriented rings with spacings that probably correspond to a unit-cell packing of 14 nm x 26 nm (Fig. 7; Wachtal, Yonath and Wittmann, unpublished).

Under appropriate crystallization conditions nucleation centers are formed within aggregates of ribosomal subunits during the initial period of crystallization[22]. An attempt to increase the size simultaneously with the order of the crystals by slowing down the crystallization process has failed, probably due to deterioration of the ribosomes before they began to aggregate. However, significantly larger and relatively well-ordered crystals have been obtained by changing the growth medium from a single solvent to mixtures of organic materials (Fig. 6).

THE NATURE OF THE INTERPARTICLE CONTACTS WITHIN THE CRYSTALS

Two-dimensional crystalline sheets have been obtained from the large subunits of B. stearothermophilus ribosomes[11] by essentially the same crystallization method used for production of three-dimensional crystals. For this purpose an active, pure preparation of the 50S subunit was used which had failed to produce three-dimensional crystals detectable by light microscopy, and further, the relative concentrations of the materials in

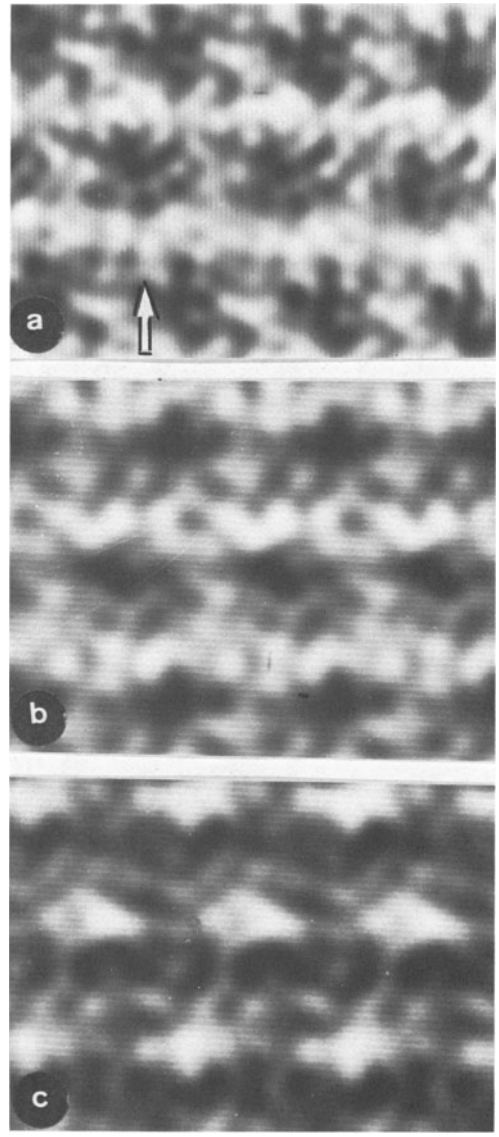

Fig. 4: Filtered images of positively stained thin sections obtained parallel to the 6_3-fold axis of crystals of 70S E. coli ribosomes. (a) (b) and (c) relate to those sections which are marked by S_0, S_1 and S_{11} in Fig. 3. The approximate direction of the 6_3-fold axis is marked.

the crystallizing medium were altered, e.g. the subunit concentration was lowered and the Mg^{++} concentration increased.

To characterize the factors that are responsible for the production of two-dimensional crystals we have investigated the formation of three-dimensional crystals in more detail as a function of the Mg^{++} concentration. 50S preparations which produce normal three-dimensional crystals at low Mg^{++} concentration were tested for crystal growth over a range of Mg^{++} concentrations. It was found that when the Mg^{++} concentration in the crystallization medium exceeds 50 mM, in most cases no three-dimensional crystals could be

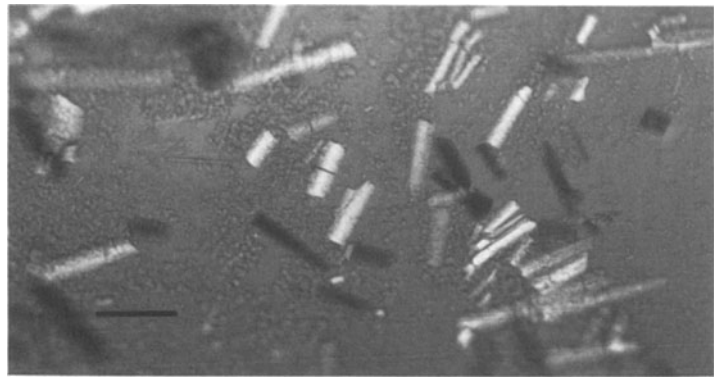

Fig. 5: Crystals of 50S ribosomal subunits of B. stearothermophilus, grown from 30% methanol at pH 8.6, as seen by polarized light microscopy. Scale bar = 0.2 mm.

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 interparticle contacts which are created in the two-dimensional plane differ from those between planes in the three-dimensional crystal. The latter are subjected to a strong competition with Mg^{++} ions whereas the intra-plane contacts are either indifferent to, or even enhanced by, the presence of Mg^{++}. The chemical properties of the two types of contact are still unknown.

The two-dimensional sheets were obtained in the presence of ethanol over the pH-range 5.8-7.2, with optimal size at pH 6.0-6.4. At lower pH-values (5.4-5.6) a slight tendency for the production of pyramid-like microcrystals has been observed (Fig. 8), and at higher pH-values (>7.5) no sheets could be detected. The three-dimensional crystals were grown in the presence of several alcohols[12] over a wide pH-range (pH 5.6-9), and those of optimal size were obtained by using 25-30% methanol at pH 7.8-8.7.

The three-dimensional crystals regularly reach their final length and width in the initial stages of crystal growth. Thus their large face is almost completely developed within 2-3 weeks. During this period the crystals are long, very thin and morphologically intact (Fig. 6). Only at later stages do they become thicker and heavier, thereby sinking to the bottom of the crystallization drop and developing cracks perpendicular to their long axes (Fig. 6). Similar fracturing occurs in cross-linked crystals, regardless of their thickness. It seems probable that the cracking and fracturing of the crystals result from mechanical stress which originates from accumulating weight or as a result of chemical forces induced by the cross-linking. For recording X-ray patterns we intended to expose single separated fragments of these fractured crystals. However, judging from the nature of the diffraction patterns (Fig. 7), it appears that we could not detect every crack by visual inspection.

It is of interest to note that all the three-dimensional crystals and two-dimensional sheets that have so far been obtained both in vivo and in vitro, are either of the large ribosomal subunits or of whole ribosomes. There have been many attempts to crystallize the small subunits, and with the exception of one case in which short helices have been reported[9], no two-dimensional sheets or three-dimensional crystals have so far been

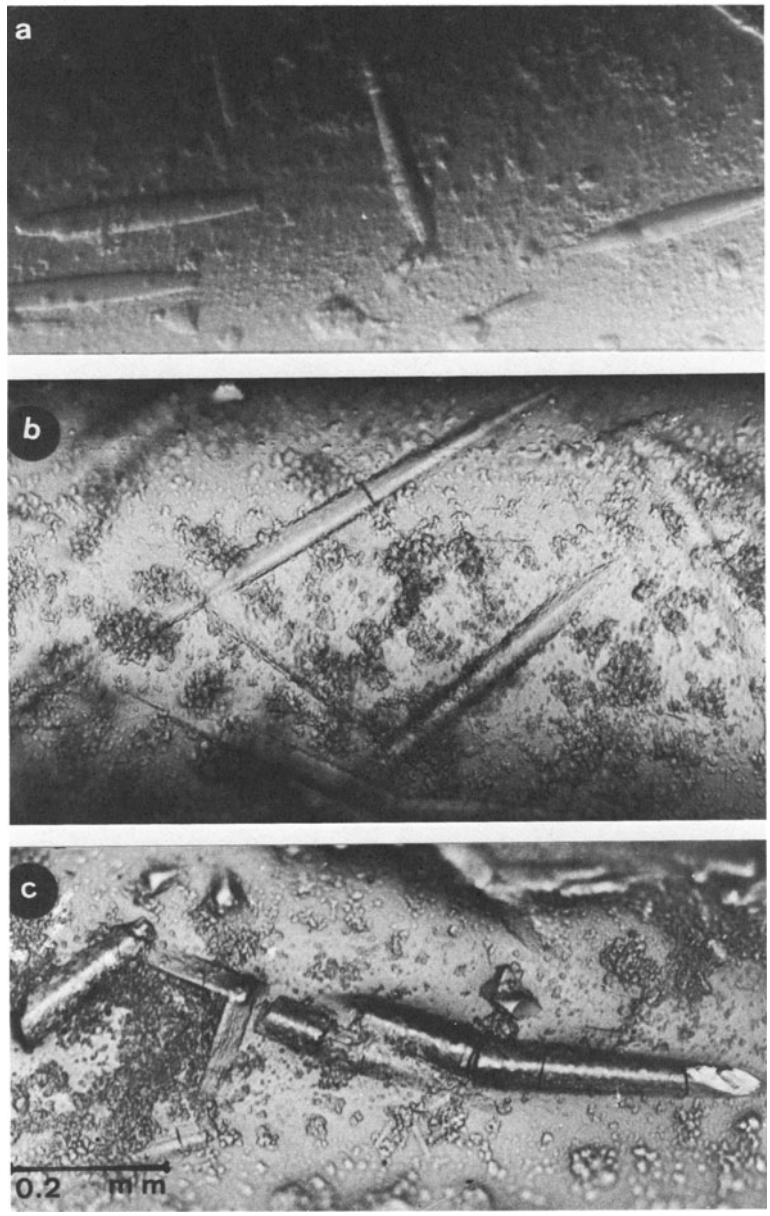

Fig. 6: Crystals of 50S ribosomal subunits of B. stearothermophilus, obtained from 30% methanol at pH 8.4 after (a) 15 days and (b) 30 days. (c) Crystals of the same particles as (a) and (b) but grown from a mixture of 17% ethylene glycol and 17% methanol at pH 8.4.

obtained. Furthermore, the interparticle contacts within the two-dimensional sheets of the whole ribosomes from the oocytes of the lizard Lacerta sicula are formed between the large subunits, and the sheets are stable even when the small subunits are removed[6]. It is not known whether this reflects an inherent flexibility of the small subunits (or a relative rigidity of the large subunits), or whether it is merely coincidence.

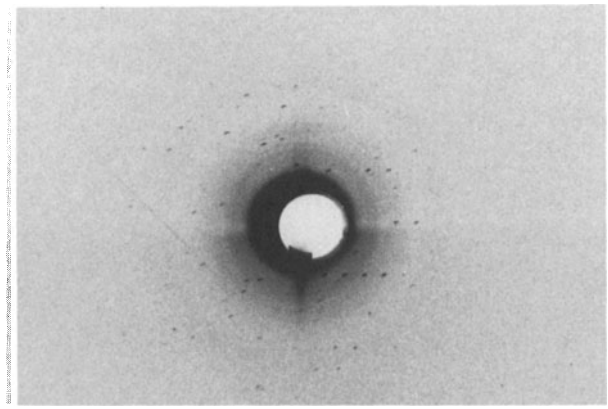

Fig. 7: The central part of a still diffraction pattern obtained with synchrotron radiation. The experimental conditions were: 5 GEV and 22-28 mA with X11/DORIS at DESY in Hamburg, 0.12 mm apperture, 12-15° C for 1 hour. The pattern is from a fragment of the crystal shown in Fig. 6(c). Some low resolution rings probably originated from microcrystals, and a precipitate that was present in the capillary can also be detected.

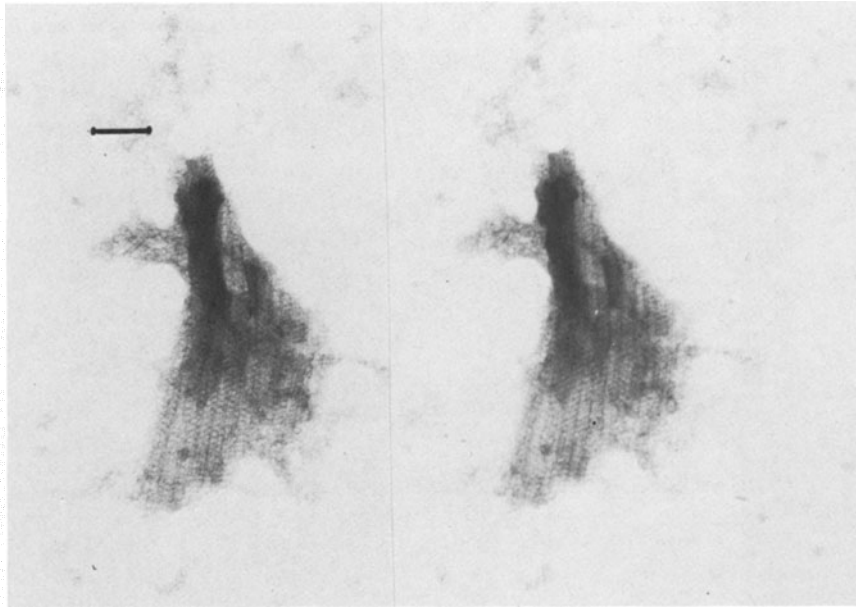

Fig. 8: The development of the growth of a three-dimensional microcrystal from a two-dimensional sheet of 50S particles from B. stearothermophilus. The negatively stained (with uranyl acetate) electron micrograph was tilted by ±10° around its axis, and the two views are shown together to enable stereo view representation. Growth conditions: 10% ethanol at pH 5.6. Bar length 100 nm.

FUTURE PROSPECTS

The ultimate goal of this line of research is to obtain a reliable three-dimensional model for the ribosome which is free from the subjective element inherent in the interpretation of electron micrographs. This model is essential for an understanding of the ribosomal role in protein biosynthesis. The attraction of the scientific problem originates from the immense biological significance of the subject. It is also conditioned by the reasonable expectation that these studies will prove to be feasible, expecially in view of the availability of suitable material, the reproducibility of crystal growth and the quality and the internal order of the crystals. Although the size of the crystals has still to be slightly improved to allow efficient diffraction studies, X-ray data collection from native crystals, using synchrotron radiation and probably newly developed detectors, is not likely to be too difficult. Fortunately, the ribosomal particles are packed in crystals with relatively small unit cells, a reasonable degree of internal order and adequate stability in the X-ray beam.

Phases whose knowledge is important for further progress may be obtained by applying one or more of the following strategies: (1) soaking the crystals in heavy atom cluster derivatives, as used for structure determination of nucleosomes[23]; (2) cocrystallizing of the 50S particles with relatively small molecules which specifically bind to the 50S subunits and to which heavy atoms can be covalently attached; (3) chemical modification of the ribosomes themselves. This might be achieved either by direct interaction of multi-metal containing compounds with the intact ribosome prior to its crystallization or by reconstitution and crystallization of particles in which protein or RNA molecules are loaded with heavy atoms; (4) using information from neutron diffraction studies[24], or from image reconstruction of two-dimensional sheets and of sections through three-dimensional crystals.

Since the ribosomes from "regular" bacteria may disintegrate at high salt concentrations, they have been crystallized from organic solvents. In order to overcome the inconvenience in handling such systems, alternative sources have been considered. One of these is to use halophilic bacteria which contain ribosomes with the unusual property of withstanding and being active at high salt concentrations. This might allow crystallization from salts such as ammonium sulfate instead of alcohols. Three-dimensional crystals of the large subunits of ribosomes from halophilic bacteria have recently been obtained[25].

Much effort and sophistication are still required before the goal of these studies, namely the elucidation of the structure of the ribosome at the molecular level, can be reached. However, the rapid progress and the very promising results achieved so far suggest that this goal is by no means unattainable.

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