In Vitro Crystallization of Ribosomes from Chick Embryos

R. A. MILLIGAN and P. N. T. UNWIN
*Department of Structural Biology, Stanford University School of Medicine, Stanford, California 94305;
and Medical Research Council, Laboratory of Molecular Biology, Cambridge, England*

ABSTRACT   A new two-dimensional ribosome crystal, having the tetragonal space group P42$_1$2 (a = 593 Å), has been grown from ribosome tetramers extracted from hypothermic chick embryos. It is of particular interest because of its larger size (up to 3 × 3 μm^2) and greater stability compared to other related polymorphic forms, and because it can easily be grown in large amounts. X-ray diffraction shows the order in the crystal to extend to a resolution of at least 60 Å. The crystalline ribosomes appear to contain a full complement of small and large ribosomal subunit proteins and an additional four proteins not characteristic of chick embryo polysomes.

A good understanding of the molecular mechanisms involved in protein synthesis will require a detailed crystallographic analysis of the structure of the ribosome by high resolution electron microscopy or by x-ray diffraction. Crystalline arrays of ribosomes have been discovered in a number of different organisms (e.g., 3, 13, 16), but none of these are sufficiently well-preserved or extensive, when isolated, for a useful investigation of fine features by either technique. The small crystalline arrays of procaryotic ribosomes and subunits which have been grown artificially (6, 7, 20) are potentially of more value.

We report here conditions that we determined for growing large crystalline sheets of eucaryotic ribosomes, derived from chick embryos subjected to cold treatment. Several polymorphic forms have been produced which are related to those found in vivo (4) and in cell extracts (1). We describe a new form, a two-dimensional crystal having the tetragonal space group P42$_1$2, that is more ordered and extensive than the others, and should be suitable for deriving accurate structural information by electron microscopy and diffraction.

MATERIALS AND METHODS
Chemicals and Solutions

All chemicals were reagent grade unless otherwise stated. Spermine tetrahydrochloride, HEPES, dithiothreitol (DTT), aspartic acid, EDTA, polyethylene glycol (PEG), Pepstatin, phenylmethylsulphonyl fluoride (PMSF), and gold thioglucose were obtained from Sigma Chemical Co. (St. Louis, MO). Ultra pure sucrose was obtained from Schwarz/Mann (Mountain View Ave, Orangeburg, NY). Unless otherwise stated, all steps in the isolation and crystallization were carried out at 4°C.

The solutions used were the following: A: 10 mM HEPES, 50 mM KCl, 10 mM MgCl$_2$, 1 mM NaN$_3$, 0.5 mM EDTA, 1 mM DTT, pH 7.2; B: the same as A, but containing 0.25 M sucrose, 1 μg/ml Pepstatin, and 100 μM PMSF; C: 10 mM HEPES, 2 M sucrose, 50 mM KCl, 5 mM MgCl$_2$, 1 mM NaN$_3$, 0.5 mM EDTA, 1 mM DTT, pH 7.2; D: 10 mM HEPES, 400 mM KCl, 5 mM MgCl$_2$, 1 mM NaN$_3$, 0.5 mM EDTA, 1 mM DTT, pH 7.2; and E: 8% PEG 20,000, 10 mM HEPES, 120 mM KCl, 1 mM MgCl$_2$, 1 mM spermine tetrahydrochloride, 1 mM NaN$_3$, 0.5 mM EDTA, 1 mM DTT, pH 7.2.

Isolation and Crystallization

Typically, 15 dozen fertile chicken eggs were incubated for 5 d and then cooled at 4°C for 12 h. Ribosomes were extracted by a modification of the method of Morimoto et al. (14).

Embryos were removed from the eggs and washed thoroughly with solution A. The washed embryos were homogenized in an equal volume of solution B in a loose-fitting teflon/glass homogenizer. The homogenate was centrifuged at 9,000 g for 5 min and the pellet reextracted as before. The first supernatant and the rehomogenized pellet were centrifuged at 9,000 g for 15 min and the resultant supernatants pooled. 20 ml of solution C and sufficient solution B were added to make a total volume of 100 ml. This postmitochondrial supernatant was layered on 10-ml aliquots of solution C in 32 ml polycarbonate tubes. After centrifugation in a Beckman Ti60 rotor at 40,000 RPM for 3.5 h, the supernatant was poured off and the clear, colorless pellets were rinsed thoroughly with solution A. The tubes were drained, and the pellets resuspended in a small volume of high salt buffer—solution D. This suspension was spun at ~12,000 g for 10 min to remove large aggregates, and the supernatant was measured spectrophotometrically. The ratio of absorbances in 12 successive preparations at 280 and 260 nm was 0.56 ± 0.01.

To determine the relative proportion of tetramers and single particles by electron microscopy, samples of the high salt-ribosome solution were sprayed with a nebulisor onto carbon-coated grids. For crystallization, ribosomes were adjusted to a concentration of 5 mg/ml (assuming 1 A$_{260}$ [1 cm path length] = 80 μg/ml ribosomes) by dilution in high salt buffer, and equilibrated, by slow dialysis, against modifications of solution E. Spectrapor dialysis membrane with a molecular weight cut off at 2,000 was used. The ribosomes crystallized over a period of 4 to 5 d.

Protein Extraction and Two-dimensional PAGE

Polysomes were isolated from 13-d-old chick embryos which had not been hypothermically treated. The isolation procedure was as described in Isolation and Crystallization, except that centrifugation was done at 40,000 rpm for 4.25 h. The pellets were resuspended in solution A at a concentration of 5 mg/ml before protein extraction. Examination of this solution in the electron microscope showed that the solution consisted of polysomes and single ribosomes. (Results not shown.)
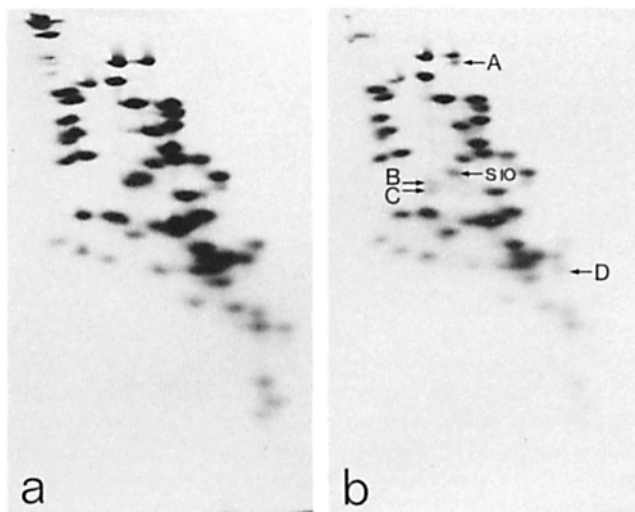
FIGURE 1 Two-dimensional PAGE patterns of basic proteins from polysome (a) and washed crystal (b) preparations. In b, the four extra proteins (A, B, C, and D) and S10 are indicated. Protein A may be a degradation product of L4—the protein spot directly above it. B, C, and S10 may be similarly related. D is in a position normally occupied by protein L29. This protein is not present in the polysome pattern. The cluster of spots close to the origin in a may represent translation factors and nascent protein as the polysomes were

Crystals were separated from mother liquor by low speed centrifugation ~2,000 g for 40 min—and washed once by resuspending in a small volume of solution A. The centrifugation was repeated. The pellet was resuspended in solution A at a concentration of ~2 mg/ml.

Proteins were solubilized and RNA was precipitated by the acetic acid method of Hardy et al. (8), as modified by Warner and Gorenstein (18). The solubilized proteins were dialyzed against 1% acetic acid, 0.2 mM DTT before lyophilization and storage at −20°C.

Two-dimensional gel electrophoresis was carried out according to Lastick and McConkey (11). A typical load was 200 μg/gel. First-dimension gels were run for 20 h at a constant 67 V towards the cathode (for separation of basic protein) or towards the anode (for separation of acidic proteins). The second-dimension gel was run for 36 h at 100 V towards the cathode. Gels were stained in 0.2% Coomassie Brilliant Blue in 50% methanol, 10% acetic acid for 6 to 8 h, and destained in 10% methanol, 7% acetic acid.

Electron Microscopy and Image Processing

Crystals in mother liquor were diluted approximately 1:100 in solution A, and carbon-coated copper grids were floated on this suspension for 60–90 s. Grids were then blotted and floated on 2% uranyl acetate solution for a similar period of time. When gold thioglucose was used as a stain, the crystals were fixed before staining by floating the grid on a solution of 0.1% glutaraldehyde in buffer A for

prepared under conditions where these would be retained (2, 19). These spots are absent or greatly reduced in intensity in the crystal protein pattern. Gels of acidic protein from crystals and polysomes give identical results in terms of ribosomal proteins (results not shown).
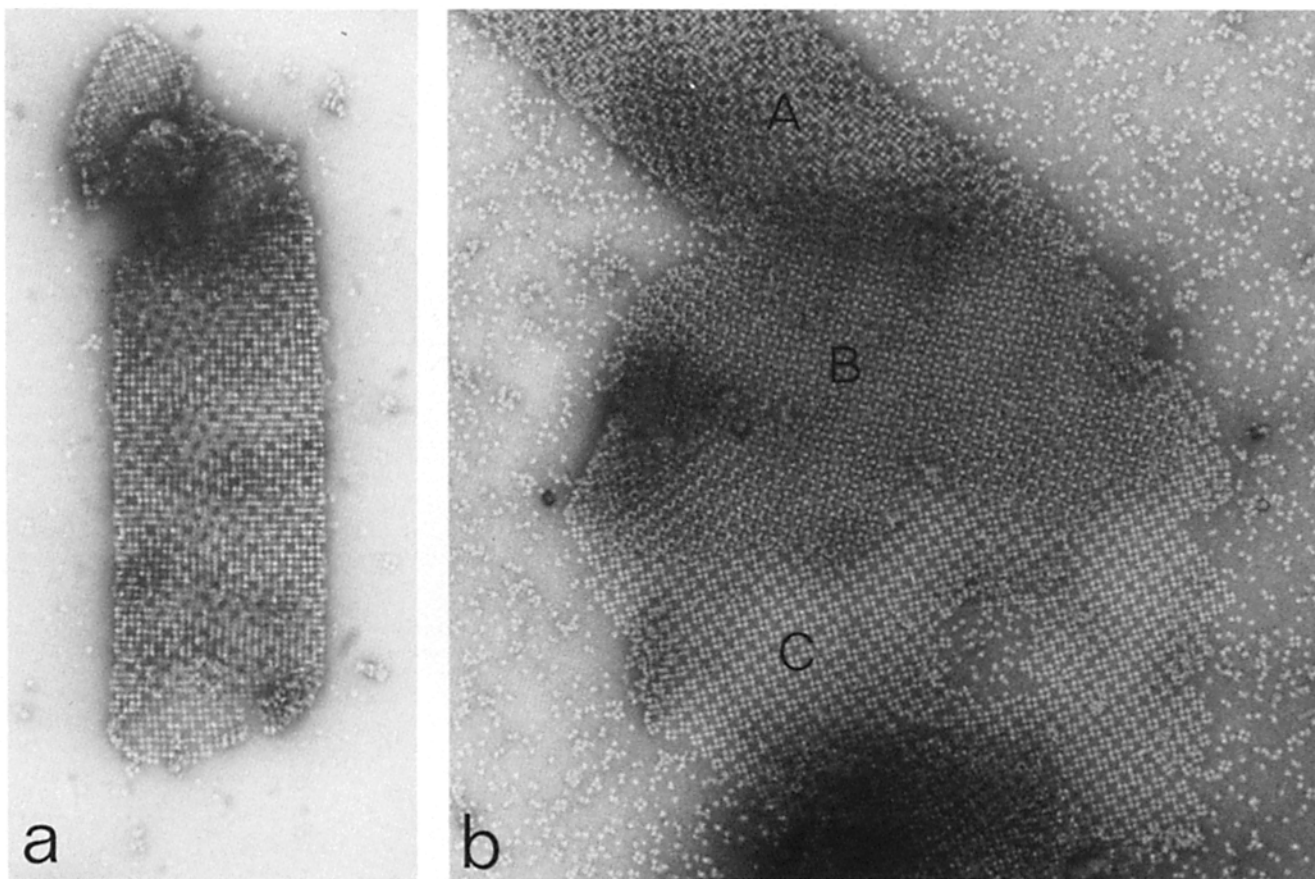

FIGURE 2 Crystal forms produced by equilibration of high salt ribosome solution against solution E without spermine. Uranyl acetate stain. (a) The predominant crystal form under these conditions is the cylinder formed from a single P4 layer. This flattened cylinder measures $0.75 \times \sim 2 \ \mu m^2$. Small portions of each side can be seen at either end of the cylinder. In the central region the two sides have collapsed onto the grid, resulting in a Σ 5b crystal (17). × 50,000. (b) An infrequently observed mixed crystal showing cylinder (A), P42₁2 crystal (B), and single P4 layer (C). The packing of tetramers is evident in the single layer which is in the right-hand configuration. × 48,000.

90 s. Images were recorded, under conditions of minimum irradiation, at a nominal magnification of 19,500 with a Philips EM400 operating at 80 kV.

Micrographs were examined by optical diffraction and areas showing best specimen preservation, in terms of resolution and symmetry present in the diffraction patterns, were selected for further processing. These areas were digitized with a Perkin-Elmer microdensitometer (Perkin-Elmer Corp., Instrument Div., Norwalk, CT) with a spot size of $25 \times 25 \mu m^2$. The array size was 512×512, each step also being 25 μm. This corresponds to a resolution of ~12 Å at the specimen. Fourier transformation of the arrays and further processing of the data were carried out essentially as described in Unwin and Taddei (17). The projection map (see Fig. 5) was calculated from peak amplitude and phase data with no symmetry imposed.

X-ray Diffraction

Specimens were prepared for x-ray diffraction by centrifugation of the crystalline suspension at ~3,000 g for 10 h onto a flat mylar sheet. The resultant pellet was composed of crystals oriented with their flat face lying predominantly parallel to the mylar surface. To facilitate handling of the pellet, it was lightly fixed with 0.1% glutaraldehyde in buffer A for 12 h. It was mounted in a wet cell so that the flat faces of the crystals were parallel to the incident x-ray beam and maintained wet and cool (4°C) throughout the exposure (1–5 d).

The x-ray source was an Elliot GX13 rotating anode x-ray generator with a

1.0 mm × 100 μm focal cup, and the camera was of the Franks type, incorporating two 22-cm glass mirrors. Patterns were recorded on CEA reflex film and developed in full strength Kodak D19 for 6 min. Film was digitized on a Perkin-Elmer microdensitometer and optical densities along the arcs and lying within a 40° sector centered on the equator were averaged using a program written by D. Austen (Stanford University).

RESULTS

Protein Composition

Electron microscope examination of the high-salt ribosome solution prepared as described in Isolation and Crystallization shows that ~50% of the particles observed exist as tetramers, the remainder consisting of monomeric ribosomes and subunits. Dialysis of this solution against modifications of solution E produces several different crystal types depending on the salt concentration, pH, and presence or absence of spermine. A comparison between the two-dimensional gel electrophoresis patterns of the crystalline ribosomes and chick embryo polysomes is given in Fig. 1. The two preparations give similar overall patterns. The major differences are the occurrence of
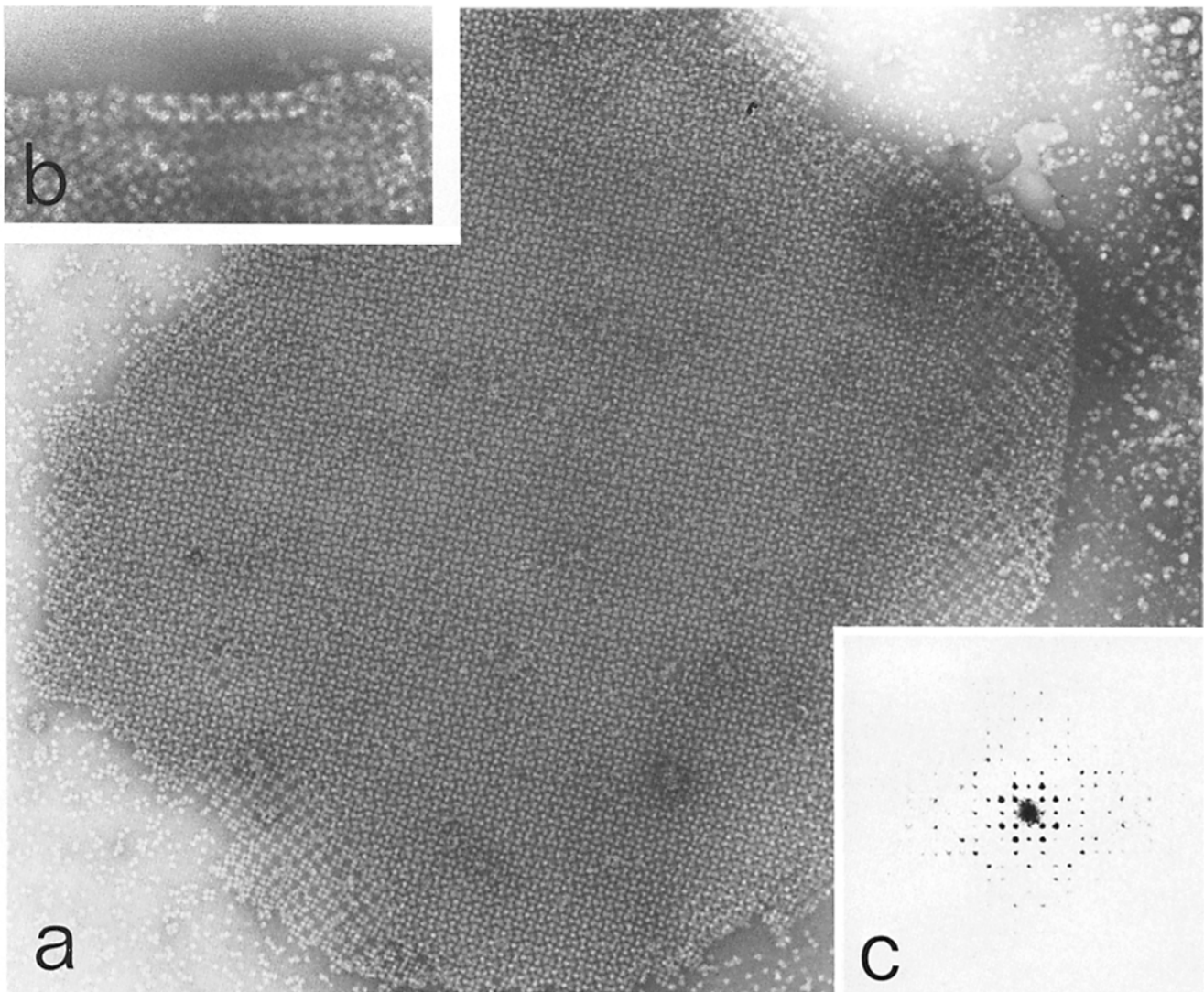

FIGURE 3 (a) P42₁2 crystal grown by equilibration of high salt ribosome solution against solution E containing 1 mM potassium aspartate. Portions of one of the P4 layers making up the crystal can be seen at the edges. Gold thio-glucose stain. × 40,000. (b) Edge-on view of such a crystal. The location of the tetramers in one layer opposite the spaces in the other layer gives rise to the staggered arrangement of stain excluding regions in this view. × 82,000. (c) Optical diffraction pattern of P42₁2 crystal. With the exception of the 0,1 and the diffuse intensity at 0,5 the pattern displays systematic absences in the directions of the unit cell vectors. The first order corresponds to an object spacing of 593 Å.
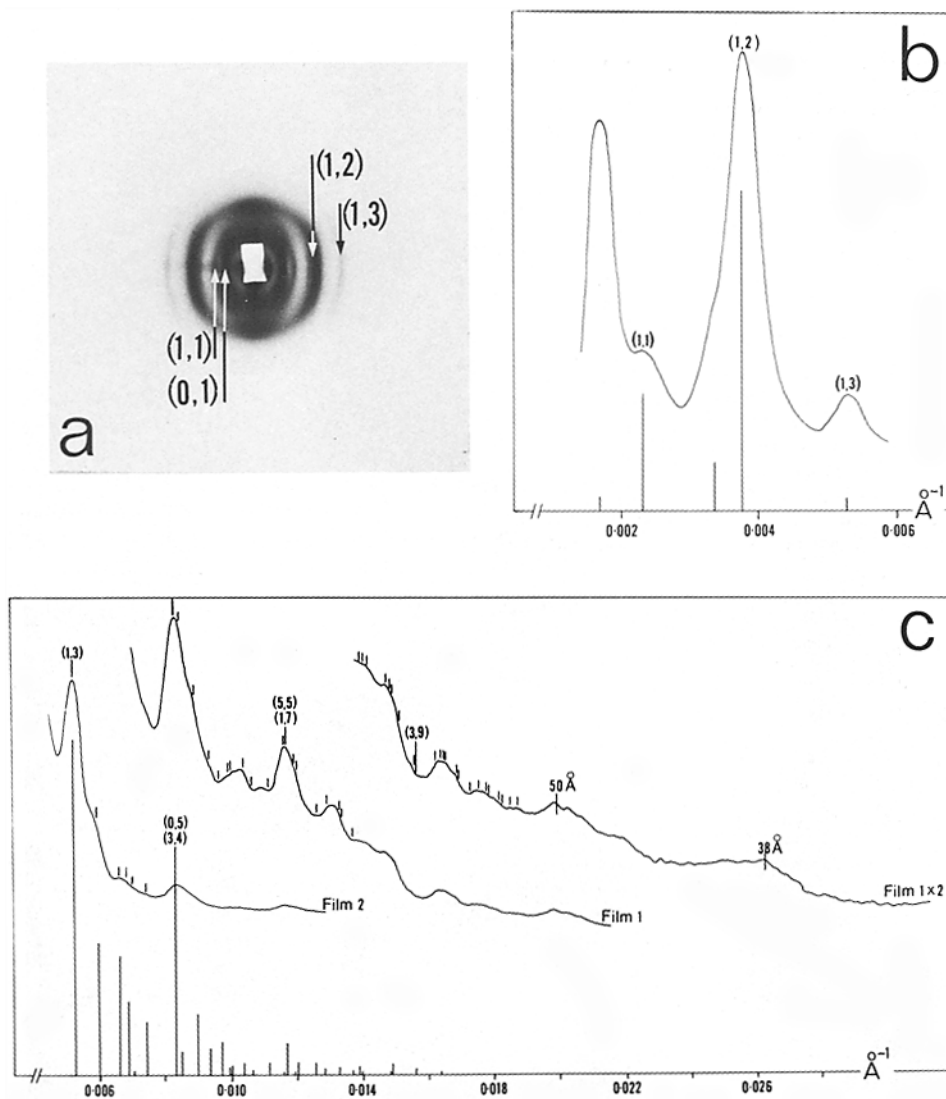
FIGURE. 4. (a) Low angle x-ray diffraction pattern of oriented crystal pellet showing equatorial arcs to the 1,3. The diffuse meridional peak is at a spacing of 1/266 Å⁻¹. Strong first-order diffraction on the equator is due to imperfect orientation of the crystals and contributions from the small proportion of crystals in the P422 and other configurations. (b and c) Comparison of relative intensities of the equatorial peaks with intensities calculated from negatively stained crystal images. The latter are represented by vertical bars on the abscissa. (c) Shows the high angle region of the diffraction pattern. The predicted positions of diffraction peaks are indicated by short vertical bars on the plot. The diffuse rings at 1/50 and 1/38 Å⁻¹ are also indicated.

four additional spots, labeled A, B, C, and D, in the crystal protein pattern. Also, in this pattern there is a decrease in the staining intensity of a protein which we have labeled S10 following the convention of McConkey et al. (12). The crystals show no evidence of significant proteolysis.

Effect of Crystallizing Conditions

All the crystal types observed are built up from a planar layer composed of ribosomes arranged as tetramers on a square, P4 lattice (Fig. 2). This P4 layer may be described as right- or left-handed (4) depending on the face from which it is viewed. Small patches of ribosomes in the P4 configuration were found under most crystallizing conditions early on in the process.

Dialysis of the ribosome solution against solution E without spermine or at pH in the range 6.5–6.9 resulted in the P4 layer curving on itself to form a closed cylinder. These cylinders are similar to those observed in vivo when embryos are subjected to very slow rates of cooling (4). The cylinders, when adsorbed to grids and negatively stained, become flattened to form rectangular sheets 0.75 μm wide and up to 3 μm long (Fig. 2). Superposition of the two sides of the cylinder, which in projection have opposite hands, gives rise to complex moiré patterns.

Dialysis of the ribosome solution against solution E with the addition of 1 mM potassium aspartate led to the formation of

sheets which are composed of two oppositely facing P4 layers. These sheets grow to a maximum size of 3 × 3 μm² and are stable for several weeks in mother liquor, or if stored in a small quantity of buffer A. They do, however, aggregate with time. A minor portion of these sheets (<20%) is constructed according to the space group P422 (4), with the centers of the tetramers in the two layers directly opposite one another. A still smaller portion is constructed according to the larger unit cell lattices found in the S. Italian lizard (17). The predominant crystal type, accounting for >80% of the crystals, has the tetramers composing one layer opposite the spaces between the tetramers in the other. This "staggered" packing, which can be deduced from inspection of electron micrographs (Fig. 3), gives the crystal a distinctive pebbly appearance.

Two-dimensional P42₁2 Crystals

Optical diffraction patterns of the latter crystals (Fig. 3) extend strongly to about the tenth order in negative stains (a resolution of 59 Å). To this resolution and along the directions of the tetragonal unit cell vectors they display systematic absences at odd values of h and k. There are weak intensities at the indices 0,1 and 0,5. However, the strength of these peaks is highly variable, suggesting that they arise artifactually, e.g., from unequal staining throughout the crystal thickness. Given
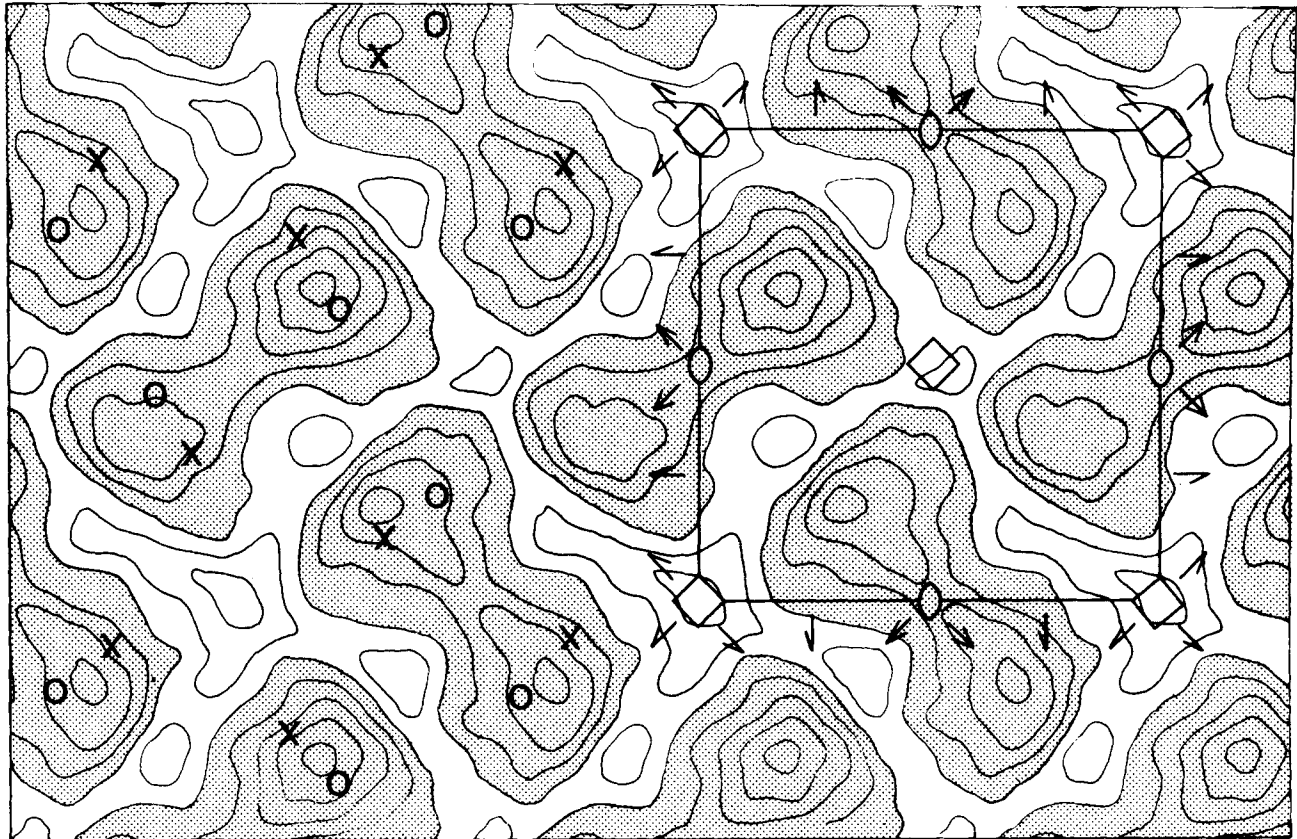
FIGURE 5 Projection map of P42₁2 crystal calculated from specimen negatively stained with uranyl acetate. The F(0,0) term was not included in the Fourier synthesis, and no account was taken of the symmetry relationships of the crystal. Refinement of the Fourier terms assuming P4 symmetry gives an average phase error of 26°, based on 32 comparisons. The shaded regions represent high concentrations of RNA and protein, i.e., regions where the stain is partially excluded. Because of superposition effects it is not possible to define the outline of a ribosome in either layer. The putative P42₁2 symmetry relationships are indicated. On the left half of the map, crosses (X) indicate the approximate center of ribosomes in the right hand P4 layer and circles (O) those in the left hand P4 layer.

the detail in the optical diffraction patterns and the observed face-to-face, staggered arrangement of the two P4 layers, the two layers of the crystal must be related both by twofold screw axes and by dyads lying in the central plane. The space group is therefore P42₁2.

X-ray diffraction patterns were recorded from partially oriented pellets of these crystals. With the plane of the sheets predominantly parallel to the beam they show a series of discrete arcs along the equator and diffuse intensities along the meridian (Fig. 4). The arcs along the equator, which arise from the crystalline packing within the plane of the sheet, index in accord with a square unit cell of dimension, a = 593 Å. They extend to a reciprocal spacing of at least $1/60$ Å^{-1}, at which point the arcs become difficult to resolve from one another at the 0.25 μm line-to-line resolution of the x-ray camera. The strong meridional peak at a reciprocal spacing of $1/266$ Å^{-1} must correspond to the center-to-center spacing between layers, since thin sections through the pellets show the sheets to be essentially randomly separated in this direction. We also observe two rather diffuse, rotationally uniform, rings of intensity, centered at reciprocal spacings of $1/50$ and $1/38$ Å^{-1}.

In comparing the lattice peak intensities for the x-ray pattern with the Fourier transform intensities calculated from electron micrographs (Fig. 4), we observe a reasonably good correspondence between the two sets of data. Thus the projection map, based on structure factors determined from electron micrographs using negative stain (Fig. 5), gives a good approx-

imation to the appearance of the crystal in its native environment.

DISCUSSION

The effect of slow cooling of early chick embryos is to allow the termination of nascent polypeptides and prevent reinitiation of translation (15). A large pool of inactive, presumably homogeneous, ribosomes is thereby created. These ribosomes have a propensity to aggregate in vivo into tetramers and subsequently into cylinders and ribosome crystals of the P422 configuration (4). The tetramers have been shown to be functional in polyphenylalanine synthesis (5, 14).

By extracting the tetramers from cooled embryos and subjecting them to slow changes in salt concentration we have been able to mimic the crystal types found in vivo and create a new type of tetragonal crystal of space group P42₁2, a = 593 Å. These crystalline ribosomes appear to contain a full complement of large and small subunit proteins and in addition four other proteins not characteristic of chick embryo polysomes. They may also lack appreciable amounts of translation factors and nascent protein as suggested by the reduction in number and intensity of spots close to the origin of the crystal protein pattern (2, 19). During cooling, the release of these proteins at the end of protein synthesis and the subsequent association of the four extra proteins with the ribosomes may be important in facilitating tetramer formation and crystallization.

The low-angle x-ray diffraction pattern (Fig. 4) shows discrete arcs, corresponding to crystalline diffraction, to ~1/60 $Å^{-1}$. The diffuse rings at 1/50 and 1/38 $Å^{-1}$ may be partly composed of sets of unresolved arcs from crystalline diffraction at 11–12 and 15–16 diffraction orders, respectively. Similar broad rings at 1/50 and 1/40 $Å^{-1}$ have been observed in patterns from rat liver ribosomes (9). Langridge (10) has interpreted the ring at 1/50 $Å^{-1}$ which he obtained from rat, rabbit, and *Drosophila* ribosome gels to originate from four or five parallel RNA double helices spaced ~50 Å apart. Our results seem inconsistent with this interpretation since the rings show no evidence of orientation.

The projection map (Fig. 5) shows a negatively stained crystal after averaging over ~100 U cells. The staggered packing of the tetramers, which appears to confer extra stability on the $P42_12$ crystals, prevents the outline or the internal structure of the ribosome from being observed in projection. These details should, however, become clear from a 3-dimensional analysis. This approach, together with labeling of the crystals with translation factors and monoclonal antibodies directed against ribosomal proteins, should prove valuable in localizing the functional domains of the eucaryotic ribosome.

REFERENCES

1. Barbieri, M. 1979. Ribosome crystallization in homogenates and cell extracts of chicken embryos. *J. Supramol. Struct.* 10:349–357.
2. Blobel, G. 1972. Protein tightly bound to globin mRNA. *Biophys. Biochem. Res. Commun.* 47:88–95.
3. Byers, B. 1966. Ribosome crystallization induced in chick embryo tissues by hypothermia. *J. Cell Biol.* 30:C1–6.
4. Byers, B. 1967. Structure and formation of ribosome crystals in hypothermic chick embryo cells. *J. Mol. Biol.* 26:155–167.
5. Byers, B. 1971. Chick embryo ribosome crystals: analysis of bonding and functional activity in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 68:440–444.
6. Clark, M. W., M. Hammons, J. A. Langer, and J. A. Lake. 1979. Helical arrays of *Escherichia coli* small ribosomal subunits produced in vitro. *J. Mol. Biol.* 125:507–512.
7. Clark, M. W., K. Leonard, and J. A. Lake. 1982. Ribosomal crystalline arrays of large subunits from *Escherichia coli. Science (Wash. D. C.).* 216:999–1001.
8. Hardy, S. J., C. G. Kurland, P. Voynow, and G. Mora. 1969. The ribosomal proteins of *Escherichia coli.* I. Purification of the 30S ribosomal proteins. *Biochemistry.* 8:2897–2905.
9. Klug, A., K. C. Holmes, and J. T. Finch. 1961. X-ray diffraction studies on ribosomes from various sources. *J. Mol. Biol.* 3:87–100.
10. Langridge, R. 1963. Ribosomes: a common structural feature. *Science (Wash. D. C.).* 140:1000.
11. Lastick, S. M., and E. H. McConkey. 1976. Exchange and stability of HeLa ribosomal proteins in vivo. *J. Biol. Chem.* 251:2867–2875.
12. McConkey, E. H., H. Bielka, J. Gordon, S. M. Lastick, A. Lin, K. Ogata, J. P. Reboud, J. A. Traugh, R. R. Traut, J. R. Warner, H. Welfle, and I. G. Wool. 1979. Proposed uniform nomenclature for mammalian ribosomal proteins. *Mol. Gen. Genet.* 169:1–6.
13. Morgan, R. S., and B. G. Uzmann. 1966. Nature of the packing of ribosomes within chromatoid bodies. *Science (Wash. D. C.).* 152:214–216.
14. Morimoto, T., G. Blobel, and D. D. Sabatini. 1972. Ribosome crystallization in chicken embryos. I. Isolation, characterization, and *in vitro* activity of ribosome tetramers. *J. Cell Biol.* 52:338–354.
15. Morimoto, T., G. Blobel, and D. D. Sabatini. 1972 b. Ribosome crystallization in chicken embryos. II. Conditions for the formation of ribosome tetramers in vivo. *J. Cell Biol.* 52:355–366.
16. Taddei, C. 1972. Ribosome arrangement during oogenesis of *Lacerta sicula* Raf. *Exp. Cell Res.* 70:285–292.
17. Unwin, P. N. T., and C. Taddei. 1977. Packing of ribosomes in crystals from the lizard *Lacerta sicula. J. Mol. Biol.* 114:491–506.
18. Warner, J. R., and C. Gorenstein. 1978. The ribosomal proteins of *Saccharomyces cerevisiae. Methods Cell Biol.* XX:45–60.
19. Wool, I. G., and G. Stöffler. 1974. Structure and function of eukaryotic ribosomes. *In* Ribosomes. M. Nomura, A. Tissieres, and P. Lengyel, editors. Cold Spring Harbor Monograph Series. 417–460.
20. Yonath, A. E., J. Müssig, B. Tesche, S. Lorenz, U. A. Erdmann, and H. G. Wittman. 1980. Crystallization of the large ribosomal subunits from *Bacillus stearothermophilus. Biochemistry International.* 1:428–435.