Journal of Supramolecular Structure 10:349–357 (1979)

Ribosome Crystallization in Homogenates and Cell Extracts of Chick Embryos

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Ribosome microcrystals have been obtained for the first time in homogenates and extracts of chick embryos mainly in the form of P422 stacks that have average linear dimensions some 40% greater than those obtained in vivo.

Key words: ribosomes, crystallization, hypothermia, chick embryos, reconstruction from electron micrographs

Ribosome microcrystals have been reported to occur either as aggregates of helices in chromatoid bodies [1–5] or as two- and three-dimensional arrays of tetramers that belong respectively to the P4 and P422 symmetry groups [6–10].

Both helical and tetrameric kinds of crystals have been found in heterogeneous systems and are formed either naturally or under the stimuli of various treatments, but in all cases they have grown exclusively within cells. This natural limitation has prevented the obtaining of microcrystals with suitable dimensions for X-ray diffraction. However, by the development of reconstruction techniques from electron micrographs [11, 12] an alternative approach to their structural study has been provided because these techniques can usefully exploit crystals of much smaller dimensions that those required by X-ray diffraction.

It appeared, therefore, that the reconstruction of the ribosome structure could be attempted from electron micrographs of the intracellular microcrystals; for this reason a number of studies have been devoted to their isolation and purification [13–15] but the resolution of the optical diffraction of the electron micrographs turned out to be disappointingly low [16].

Reconstructions of three-dimensional models of the ribosome have been performed from the helical arrays of the chromatoid bodies [17, 18] and from the tetrameric microcrystals of lizard oocytes [19, 20] by exploiting both optical and electron diffraction techniques and by the rigorous reconstruction approach of the Fourier method, but the

Received February 21, 1979; accepted February 21, 1979

resolution limits that could be achieved were, respectively, 107 Å and 90 Å, and at these levels even the reconstruction of the gross configurations of the ribosome subunits turned out to be problematic.

In this situation it appeared that a suitable alternative approach consists in overcoming the fundamental limitation represented by the growth of the ribosome crystals within the cell environment and in obtaining crystals of greater dimensions than those that are formed within the cell. This study describes a first group of experiments that show that this approach is a practicable one. For the first time crystallization of eukaryotic ribosomes outside the cell has been achieved. Although crystals of suitable dimensions for X-ray diffraction have not yet been obtained, it is possible that further improvements may eventually allow that goal to be reached.

METHODS

Isolation and Crystallization Procedures

Fertile chicken eggs of 5 or 5½ days of incubation were transferred to the cold room, where the embryos were quickly removed and immersed in ice-cold TKM medium A (50 mM Tris, 300 mM KCl, 5 mM $MgCl_2$; pH 7.6 at 20°C). After removal of the amnion the embryos were washed, often beheaded, and then transferred to a cylinder containing TKM Medium B (0.2 M sucrose in Medium A) whose volume was adjusted to give a 1:1 (v/v) final dilution of the tissue. Between 180 and 200 embryos were used for a standard experiment and homogenized with ten strokes of a motor-driven Teflon-glass homogenizer. In the first experiments debris was removed from the homogenates with a 500 g centrifugation for 5 min, but later this step was omitted as unnecessary.

Cell extracts were obtained by centrifuging a homogenate in the SW 27 rotor of a Beckman ultracentrifuge at 3,000 rpm for 10 min and the resulting supernatant at 12,000 rpm for 15 min. The top half of the final supernatant was recovered and referred to as the postmitochondrial extract or the PM fraction. Both the nuclear and the mitochondrial pellets were resuspended in the remaining supernatant and homogenized again, giving a reconstituted and concentrated homogenate that was referred to as the enriched-homogenate fraction. When necessary, the ribosomes of the PM fraction were concentrated by centrifuging the postmitochondrial supernatant at 25,000 rpm for 5–6 h and then resuspending the pellet in what remained after the removal of the top nine-tenths of the supernatant.

The unfractionated homogenate or the cell extracts obtained from it were each poured into a glass cylinder up to the brim and sealed with a plastic cork in such a way that no air was trapped inside. The cylinders were incubated at 38°C in a water bath for 30 min, then wrapped in several layers of tissue paper or cheesecloth and transferred back to the cold room, the purpose of wrapping being that of producing a gradual (but not critical) decrease of temperature.

The wrapped cylinders were attached to a rotor that kept them revolving in a plane perpendicular to the floor at 2 rpm for 48 h at 4°C. After that, the solutions were diluted by 20% with buffer A, usually containing 5% Triton X-100 and were homogenized and centrifuged immediately in the SW 27 rotor of a Beckman ultracentrifuge.

The sediments obtained after centrifugation at 3,000 rpm for 10 min, 10,000 rpm for 20 min, and 20,000 rpm for 30 min were referred to as the 3k, 10k, and 20k pellets, respectively.

Electron Microscopy

Pellets were fixed in 0.1 M sodium cacodylate buffer (pH 7.6) containing 2.5% glutaraldehyde for 6–8 h at 4°C, rinsed in buffer for 12–16 h at 4°C, postfixed in 1% osmium tetroxide at room temperature for 1 h, dehydrated in an acetone dilution series, and finally embedded in Epon. Sections were cut on a Reichert Ultramicrotome, stained with uranyl acetate and lead citrate at room temperature, and observed under a Siemens Elmiskop 1A electron microscope.

For statistical purposes, the square region of the hole of a 300-mesh grid was assumed as a unitary area of which serial photographs were taken at 5,000 magnification, their enlarged prints being composed to give a 1-m^2 picture of each area. The computations were done by assuming as linear dimension of a microcrystal the length of the maximum segment that could be measured within the crystal.

RESULTS

When homogenates of chick embryos were hypothermized and then fractionated as described in Methods, ribosome microcrystals were found in all pellets with yields and average dimensions that are maximal in the 3k pellet and progressively decrease in the other fractions. Figure 1, for example, represents crystals characteristic of the 3k pellet, Figures 2 and 3 crystals characteristic of the 10k pellet, and Figure 4 crystals characteristic of the 20k pellet.

All crystals appear to have the symmetry of those grown in vivo and display therefore the classic P4 arrangement of tetramers when sectioned along the planes of their constituent layers (Fig. 3), while in sections perpendicular to these planes (Figs. 1, 2, 4, and 6) each layer appears as a row of ribosomes which form dotted or dashed sequences of particles depending upon the orientation of the line of sectioning in the plane of the crystal layer, as described in a previous report [23]. The largest microcrystals (3–6 μ) frequently have a polycrystalline structure (Fig. 5), but those of intermediate dimensions (1–3 μ) were often found as P422 monocrystals (Figs. 1, 2, and 6).

There are some significant differences between the crystals grown in vivo and in cell fractions. While in vivo the great majority of the microcrystals consist of two-dimensional P4 layers, those obtained in homogenates are predominantly P422 stacks of such layers, and the average number of layers per stack is some 50% higher than that of the intracellular crystals. Furthermore, the microcrystals formed in homogenates had average linear dimensions some 40% larger than those obtained in vivo, even when these were isolated by various methods [13–15] and equivalent pellets were compared.

The yield of microcrystals obtained from homogenates was compared with that obtained by standard cooling of embryonated eggs using two methods. The first consisted of disaggregating the crystals, isolating the tetramers that derive from them on density gradients using established procedures [21, 22], and assuming that the quantitative recoveries of the tetramer bands were representative of the microcrystal yields. The second was an electron microscopy analysis of the pellets of equivalent subcellular fractions carried out by the semiquantitative procedure described in Methods. Both procedures showed that the yields of microcrystals from homogenates ranged between 60% and 70% of the yield obtained from whole embryos.

The same crystallization procedure applied on the fractions of a cell homogenate obtained as described in Methods produced the following results.

Sections prepared from the pellets of the enriched homogenates (Fig. 6) turned out to be very similar to those of the unfractionated homogenates, showing that the formation of microcrystals was not affected by sedimenting and resuspending the large fragments of

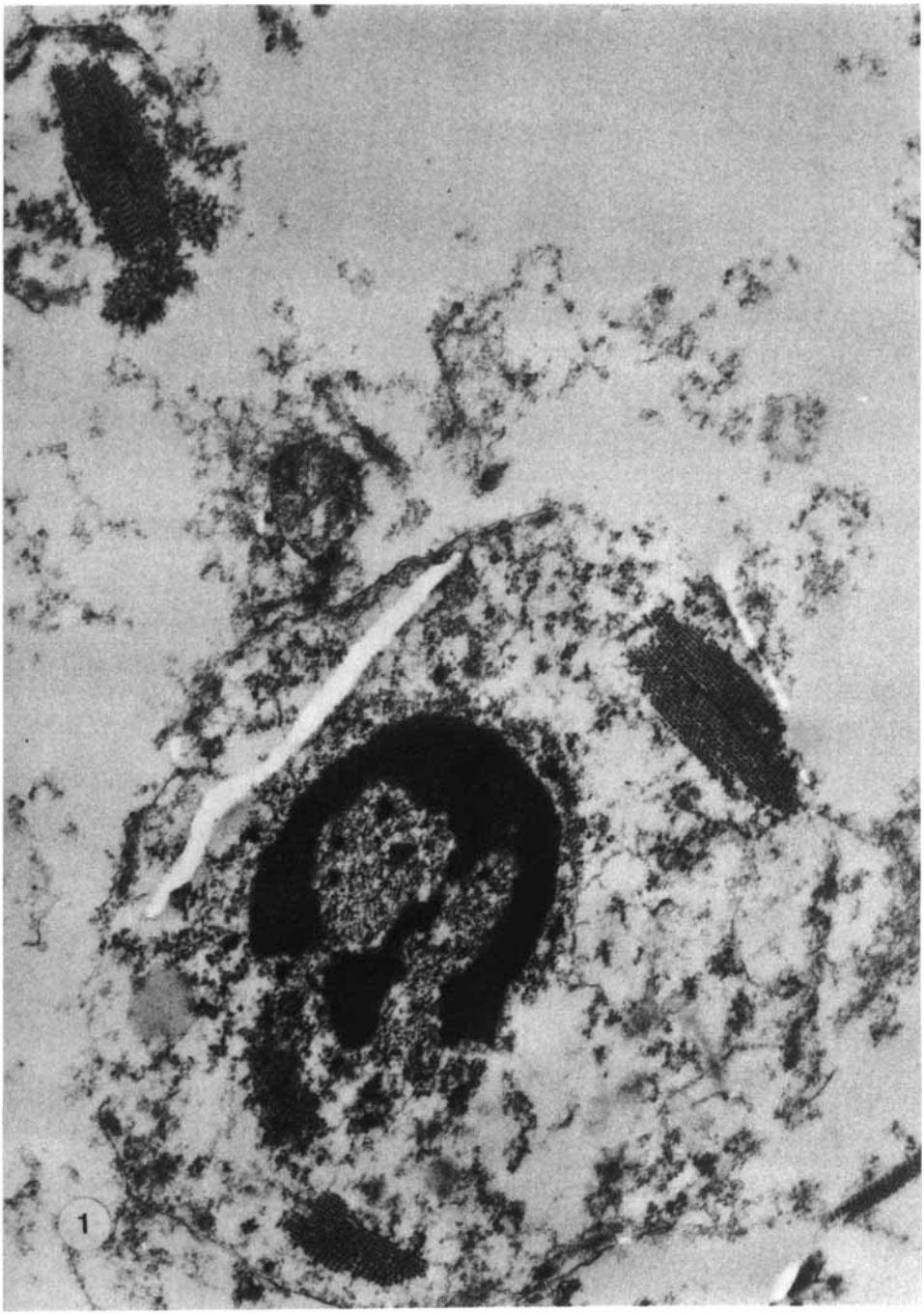

Fig. 1. Representative view of the 3k pellet of a chick embryo homogenate obtained after the crystallization procedure described in Methods. Remnants of the nuclear membrane are visible. 18,000×.

the homogenate and by carrying on the crystallization in a more concentrated solution. The PM fraction, on the contrary, showed barely detectable signs of crystallization. Figure 7 is a picture of a 20k pellet of the PM fraction, but the small crystal that appears in it represents, in fact, a very rare finding, not at all a common feature.

In order to confirm this pattern, the ribosomes of a PM fraction were added to an enriched homogenate, but again their contribution to crystallization was found to be

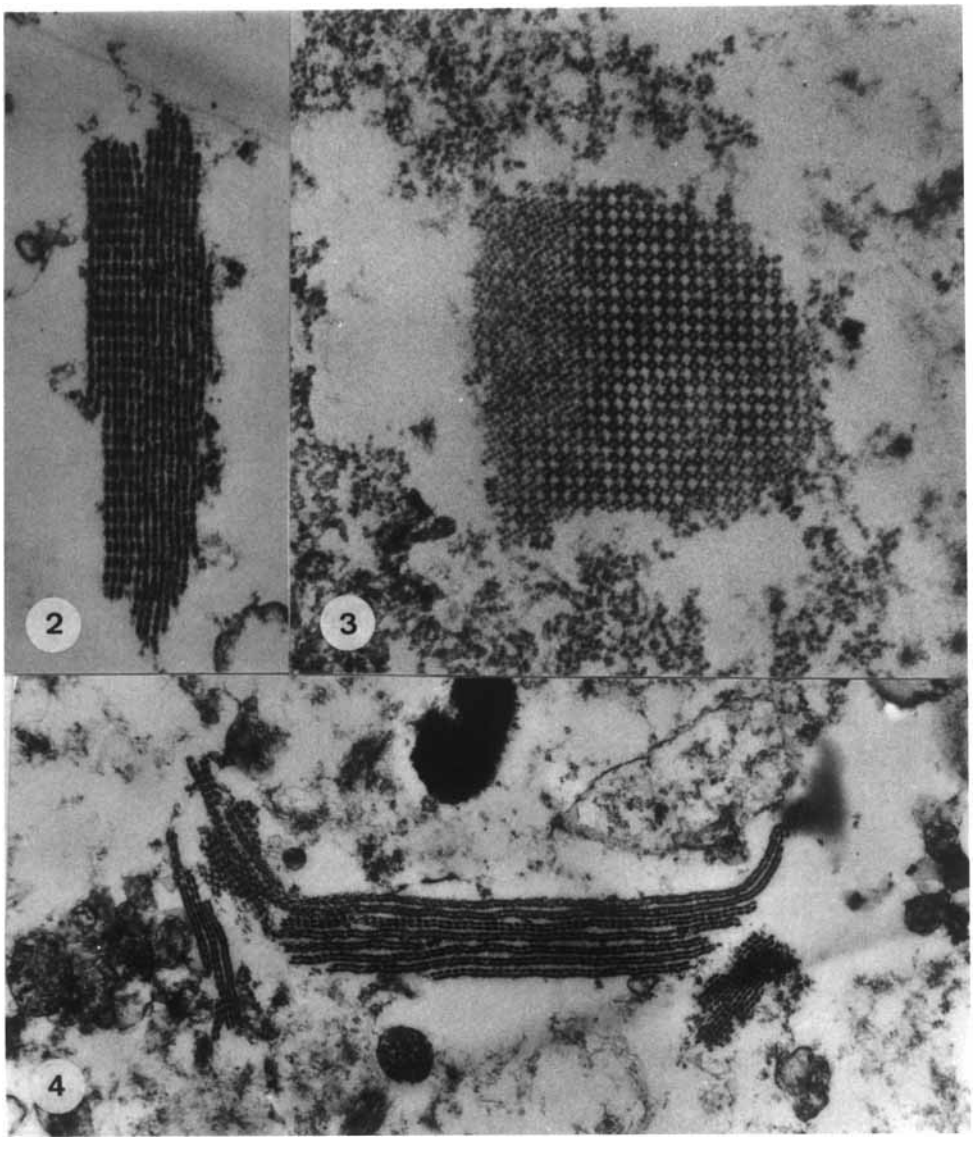

Fig. 2. Ribosome crystals of the 10k pellet in sections that are perpendicular to the plane of the P4 layers. 36,000×.

Fig. 3. Ribosome crystals of the 10k pellet in sections that are parallel to the plane of the P4 layers. 36,000×.

Fig. 4. Microcrystals recovered in the 20k pellet. 18,000×.

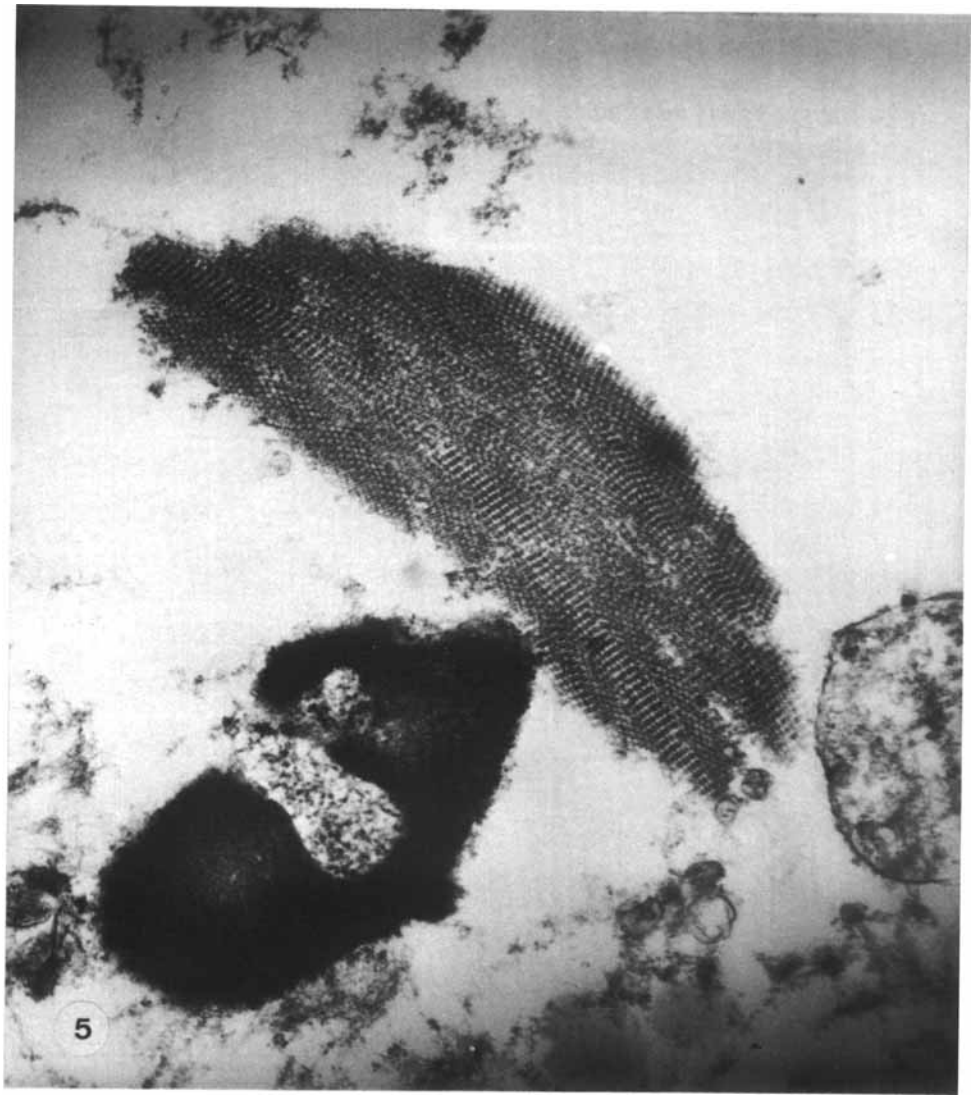

Fig. 5. Representative view of a large-size ribosome microcrystal. 21,600×.

practically negligible. The amounts of tetramers that were obtained on density gradients from enriched homogenates with an without the addition of the PM ribosomes were virtually identical.

This result is in agreement with an earlier report [24] according to which the crude nuclear and mitochondrial fractions obtained from chick embryos before and after hypothermic treatment do not have significant differences in their RNA contents, which suggests that crystallizable ribosomes are associated with heavily sedimenting structures even before crystallization takes place.

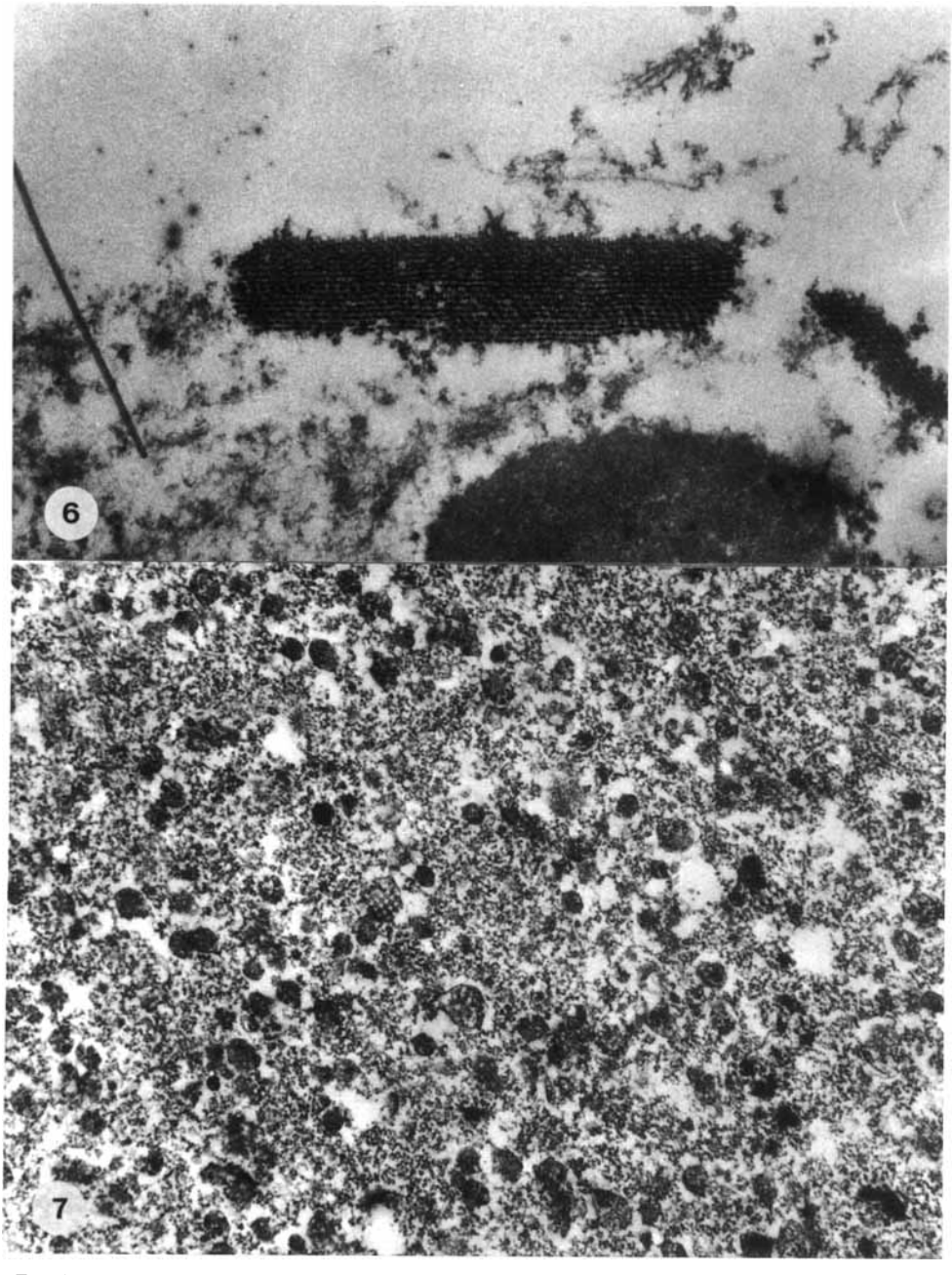

Fig. 6. Typical example of the medium-size microcrystals that are recovered in the 3k pellet of the enriched-homogenate fraction. 27,000×.

Fig. 7. Pellet of the postmitochondrial fraction submitted to the crystallization procedure described in Methods. 18,000×.

DISCUSSION

The main goal of this study was to show that the integrity of the cell is not essential for the growth of ribosome microcrystals; particular care was taken, therefore, to ensure that the results of the experiments could not receive a different interpretation.

A first objection, for example, might be that crystallization could have taken place before homogenization, but this possibility can be disregarded. In intact cells ribosome microcrystals start appearing only after three hours of cooling at 4°C, while in our case homogenization invariably took place within one hour of the transfer of the embryos to the cold room. Furthermore, in a few cases the embryos were quickly removed at room temperature and the results did not show appreciable differences. Finally, after homogenization the samples were always incubated at 38°C for 30 min and it is known that this treatment would have disaggregated any crystal which might possibly have been formed before homogenization.

A second objection, that crystallization might have taken place only in the cells that had remained intact after homogenization, can be disproved by three arguments. The first is that the experiments in which intact cells were removed by low-speed centrifugation before crystallization were equally successful as those in which debris was not removed. Second, the yields of microcrystals, evaluated by two independent methods, correspond on average to 65% of the recovery obtained from whole embryos, and the amount of cells that could have escaped disruption could in no way account for that percentage. Third, the dimensions of the microcrystals obtained from homogenates are consistently greater than those observed inside the cells. Crystals like those of Figure 5, for example, which have width and height in excess of 6 and 2 μ, respectively, have never been reported to occur in intact cells of chick embryos.

The above experiments prove, therefore, that ribosome crystallization can be obtained outside the cell; now that the obstacle of intracellular confinement is removed, it is conceivable that ribosome crystals can be grown up to dimensions that allow studies with higher resolution.

This expectation would not be realistic if it were found that the intracellular environment, although not essential, is nevertheless the optimal one for crystallization, but the experiments show that this is not the case. Although the observed increase in size of the microcrystals was only moderate, the very fact that it did take place shows that the basic assumption of the approach is sound and that one can therefore expect improvements by further refinements of the crystallization technique.

ACKNOWLEDGMENTS

I am deeply grateful to Professor H. G. Wittmann for continued discussions and support. I also wish to acknowledge the skillful help of Dr. A. W. Siddiqui for the electron microscopy preparations.

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