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Chick Embryo Ribosome Crystals: Analysis of Bonding and Functional Activity *In Vitro*

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ABSTRACT The intracellular ribosome crystals from chick embryos incubated at 5°C have been isolated; stable products of their dissociation were shown to include single ribosomes, tetramers of ribosomes, and tetramers of large subunits. Conditions were found for the formation *in vitro* not only of the *p4* crystals from ribosomal tetramers but also of the tetramers themselves from free ribosomes. The polypeptide synthesizing activity of these ribosomes was found to be unimpaired by their constraint in the tetrameric configuration.

The ribosomes of chick tissues form intracellular crystals when the embryos are incubated at low temperature (1). Although one might hope that such crystals would prove useful for structural analysis of ribosomes, the intracellular crystals are only about one micrometer in diameter and hence far too small for single-crystal x-ray diffraction. It has been the aim of the present studies to explore the characteristics of these crystals *in vitro* so that larger crystals might be grown and used for the analysis of ribosomal structure.

The intracellular crystals are principally in the form of a *p4* plane lattice, which may under certain conditions aggregate into regular stacks defined by a three-dimensional lattice of the form *p422* (2). Extraction of whole cells with a buffer suitable for the separation of polysomes reveals stable planar tetramers of ribosomes (3), many of which are derived from intracellular crystals. In the present studies, the *p4* crystals were first extracted from the cells, the nature of bonding in these crystals was then explored by dissociation and formation of their bonds *in vitro*. In addition, the synthetic capabilities of the tetramers have been analyzed with a view toward correlating structural and functional features of these ribosomes.

MATERIALS AND METHODS

All ribosomes were derived from embryonated chicken eggs incubated for 7 days at 37°C. Tetramers and crystals were produced inside the cells by gradually reducing the temperature of the eggs over a span of 4 hr to 5°C and holding the eggs at that temperature for an additional 12–16 hr. Embryos were then removed to buffer (10 mM $MgCl_2$–100 mM KCl–10 mM Tris·HCl (pH 7.4)–5 mM 2-mercaptoethanol) at 4°C and were disrupted with eight rapid strokes of a loose-fitting Dounce homogenizer. The post-mitochondrial supernatant was layered onto 10–25% (w/w) linear sucrose gradients in buffer and centrifuged for 3 hr at 25,000 rpm in the Spinco SW-27 rotor. The gradients were fractionated through a Gilford spectrophotometer and the peaks of single ribosomes and tetramers were pooled and pelleted separately through 2 M sucrose. The pellets were resuspended in a small volume of buffer and their concentrations determined by their absorbance at 260 nm.

Free ribosomes were prepared from uncooled embryos by incubating the postmitochondrial supernatant with the following added components for 5 min at 37°C, pH 7.5: ATP, 1 mM; GTP, 0.4 mM; phosphoenol pyruvate, 10 mM; Tris, 50 mM; puromycin, 4 mM. The yield of single ribosomes on gradients after puromycin treatment (8–10 mg/g wet weight of tissue) was about 10 times that on gradients of untreated extracts. A single pelleting through 2 M sucrose in normal buffer removed puromycin sufficiently that the ribosomes were fully active in poly(U)-directed polyphenylalanine synthesis.

Samples for electron microscopy were picked up on carbon-coated parlodion-covered grids, fixed for 10 min in 3% glutaraldehyde containing 5 mM $MgCl_2$ and 5 mM phosphate buffer at pH 7.5, and negatively stained with 2% uranyl acetate. In order to test the ability of free ribosomes and tetramers to form new bonds of the crystal when transferred to fresh buffers, a microdialysis method for electron microscopy was developed: for each dialysis, an electron microscope grid was coated on one side with a parlodion film and the grid was floated with the other side on a drop of the new buffer. A small volume (0.5–1.0 μl) of ribosomal suspension was pipetted onto the center of the parlodion film and the grid was incubated for about 6 hr at 5°C in a humid chamber. The sample was then rinsed with the same buffer, fixed, and stained before carbon-coating. All grids were examined in an AEI 6B electron microscope at 60 kV.

The formation of tetramers *in vitro* was assayed quantitatively by centrifugation at 4°C on 10–25% sucrose gradients in the SW 41 rotor of the Spinco L2-65B and analyzed with an ISCO density gradient analyzer. Before being layered onto gradients, samples of 0.2 mg were diluted to 0.3 ml in buffer and heated for 3 min to 37°C in order to dissociate nontetrameric aggregates; the cyclically bonded tetramers remained stable under these conditions.

Phenylalanine incorporation in the absence and presence of poly(U) was determined as described in the legend to Table 1.

RESULTS

Isolation and dissociation

Tetramers from cooled chick-embryo tissues were originally found in sucrose gradients that contained a buffer designed for

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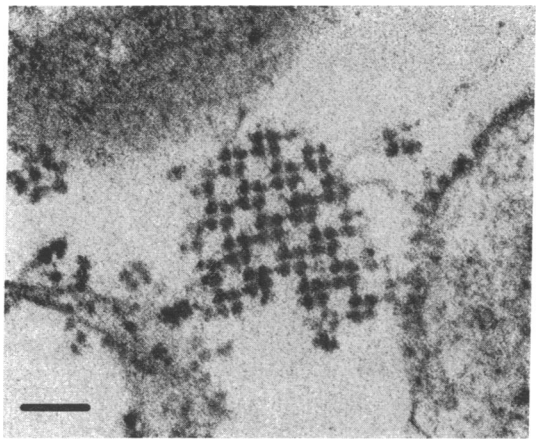


FIG. 1. Ribosome crystal isolated from an embryo incubated 6 days and cooled for 20 hr to 5°C. The pellet of crystals was fixed in phosphate-buffered glutaraldehyde, post-fixed with OsO_4, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate. (Bar = 100 nm.)

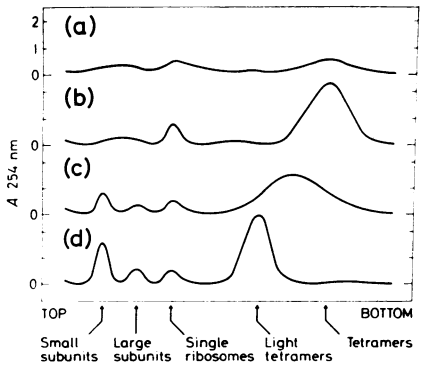


FIG. 2. Dissociation of isolated ribosome crystals in sucrose gradients with different KCl concentrations. Samples (0.4 ml) of crystals pelleted and resuspended in 10 mM $MgCl_2$–10 mM Tris·HCl, (pH 7.4) were layered onto 10–25% sucrose gradients in the same buffer plus various concentrations of KCl and centrifuged for 60 min at 40,000 rpm, 4°C, in the Spinco SW-41 rotor. Absorbance profiles at 254 nm were recorded in the ISCO density gradient analyzer during fractionation. Representative results are shown in (*a*) 0.05 M KCl, (*b*) 0.2 M KCl, (*c*) 0.4 M KCl, and (*d*) 0.8 M KCl. The peak of tetramers in (*b*) is nearer the top of the gradient in (*c*) and especially so in (*d*). This shift is greater than that of a nondissociable marker ("stuck" single ribosomes) in similar gradients. There is a concomitant increase in the size of the peak of small subunits.

the separation of single and polysomal ribosomes from developing skin: 10 mM KCl–10 mM Tris·HCl–1.5 mM Mg^{++} (4, 5). Having first confirmed that whole 7-day embryos yield tetramers as well, I tested buffers less disruptive to ribosomal aggregates for their tendency to leave the *p4* crystals intact. I found that raising the Mg^{++} concentration and lowering that of KCl preserved this bonding. In a usual preparation, slowly-cooled embryos were homogenized at 0°C in 10 mM $MgCl_2$–10 mM tris·HCl at pH 7.4, and the heavier material was removed by 5 min centrifugation at 500 × *g*. The supernatant contained *p4* crystals which could be pelleted and prepared for thin-section electron microscopy (Fig. 1).

It was then possible to investigate the sensitivity of the dimer bonds (joining tetramers together in the *p4* crystal) to the addition of KCl. Pelleted crystals were resuspended in the same buffer and layered onto sucrose gradients containing the same amount of $MgCl_2$ (10 mM) and Tris·HCl (10 mM) but with 0.05 M increments in KCl concentration (Fig. 2). At less than 0.1 M KCl, few tetramers appeared on the gradients; from 0.15 to 0.25 M KCl, there were significant numbers of tetramers present (Fig. 3*a*). At 0.30 M KCl and above, however, the "tetramer" peak shifts nearer the top of the gradient than can be accounted for by the increased density and viscosity of the added salt. This indicated, as suggested by similar studies of Carey (6), that the particles have been modified in some manner. In order to verify that the lighter material is actually derived from tetramers, samples of purified tetramers were layered onto gradients containing 0.8 M KCl; the resulting profiles showed not only a high yield of these lighter particles but also a great increase in the peak of small (40 S) ribosomal subunits. It was confirmed that the peak of lighter particles represents an aggregate of the large (60 S) subunits by further gradient analysis for the display of subunits (in 1 M KCl, no $MgCl_2$, 10 mM Tris·HCl). Whereas free ribosomes and normal tetramers from cooled tissues yielded small and large subunits in approximately equal numbers, only the large subunits were found in the "light tetramers". Similarly, agarose–acrylamide gels of phenol-extracted RNA from tetramers and "light tetramers" revealed both 18S and 28S RNA in the tetramers, but only 28S RNA in the light tetramers. Furthermore, electron microscopy of the "light tetramers" (Fig. 3*b*) shows that they retain the profile of normal tetramers but appear not to extend as far upward into the layer of negative stain. These results suggest that the normal tetramer consists of a planar aggregate of large subunits to which are attached the small subunits along axes nearly parallel to the direction of view, so that the projected views of the two subparticles are largely overlapping.

Tetramers cannot, therefore, be dissociated to single ribosomes by the simple addition of KCl because the salt separates small subunits from "light tetramers". It was found possible, however, to produce free ribosomes by shocking tetramers with 0.8 M KCl in 10 mM $MgCl_2$–10 mM Tris·HCl at 37°C for 5 min before they are separated on a gradient of

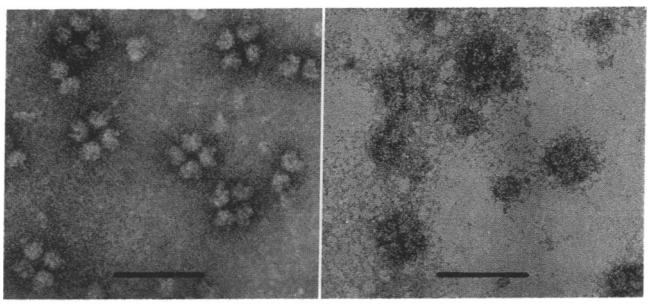


FIG. 3. Ribosomes from gradients such as those in Fig. 2 fixed in glutaraldehyde and negatively stained with uranyl acetate: (*a*) normal tetramers, (*b*) "light tetramers" of large ribosomal subunits alone. (Bar = 100 nm.)

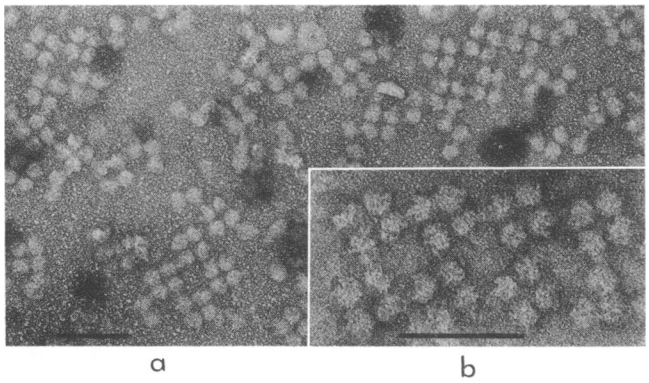


FIG. 4. Re-formation of dimer bonds of the *p4* crystal *in vitro*. Tetramers were isolated from cooled embryos and dialyzed at 5 mg/ml against 10 mM $MgCl_2$–80 mM KCl–3 mM phosphate buffer (pH 7.5) for 6 hr at 5°C before fixation and negative staining for electron microscopy as described in the text. Note that the *p4* lattices in (*a*) all show the same (*dextro*) form; they were printed to represent the view from the substrate side of the specimen. The insert (*b*) shows for comparison the other (*levo*) form, which would be seen from above the substrate. (Bar = 100 nm.)

normal buffer. These salt- and heat-shocked ribosomes were used in initial attempts to reform tetramers *in vitro*.

Bond formation *in vitro*

In order to test the ability of free ribosomes to form tetramers and of tetramers to form crystals *in vitro* after transfer to new buffers, the microdialysis method was employed. Suspensions of tetramers at 5 mg/ml were dialyzed 4–6 hr at 5°C and then prepared for electron microscopy. Dialysis buffers contained 3 mM phosphate buffer, pH 7.5, and concentrations of KCl and $MgCl_2$ determined to be near the boundary conditions for bond stability in the dissociation experiments. Best results (Fig. 4) were obtained with concentrations of $MgCl_2$ near 10 mM; in these cases, small *p4* crystals—usually containing four tetramers in square array—appeared when the buffer also contained 0.08–0.20 M KCl. At lower concentrations of KCl, there were either irregular aggregates or sets of tetramers in correctly-bonded linear arrays; at higher concentrations, there was little crystallization. A striking feature of the crystals grown *in vitro* is that all of those observed showed the same polarity with respect to the parlodion substrate. That is, if viewed from the substrate side, all were of the *dextro* form defined earlier (2). This implies that the surface that is normally concave and bounded by a denser matrix in thin-sectioned crystals lies against the artificial substrate in the dialysis experiment. The significance of this behavior remains obscure. No factors stimulatory to the growth of these very small *p4* crystals were discovered nor was a consistent dependency on ribosomal concentration determined for the process.

The same method was applied to the analysis of tetramer reformation from free ribosomes derived from salt- and heat-shocked tetramers. Tetramers were, in fact, frequently found on dialysis grids, but the kinetics appeared to be erratic, and it was soon discovered that tetramers were appearing in undialyzed suspensions of free ribosomes at high concentration in normal (as opposed to dialysis) buffer.

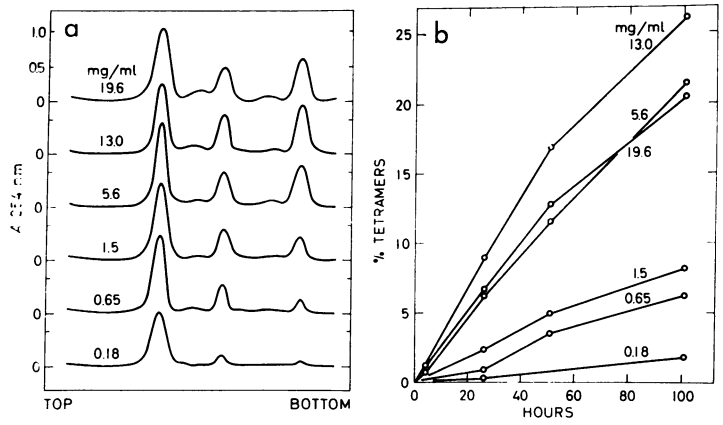
It was then possible to omit the dialysis procedure and simply follow this process by the use of analytical sucrose gradients. Suspensions were diluted in normal buffer to less than 0.8 mg/ml and heated for 5 min to 37°C before layering, in order to dissociate aggregates which were not "true" tetramers, as determined by electron microscopy. Pilot experiments revealed that the tetramer-derived ribosomes were competent in tetramer formation. Moreover, single ribosomes isolated by preparative gradients from either cooled or normal tissues were also found to be competent. In order to obtain sufficient quantities of free ribosomes, extracts of normal tissue were treated with puromycin as described in *Methods*, the ribosomes thus freed from polysomes in good yield were also found to be competent in tetramerization. These ribosomes were suspended in various buffers and incubated at 0°C or higher temperatures before gradient analysis. The resulting absorbance profiles were analyzed by planimetry to determine the relative proportions of ribosomes that had formed tetramers.

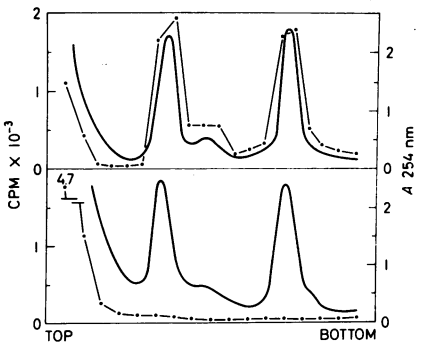
These puromycin-freed ribosomes formed tetramers to yields greater than 30% without the addition of any other components. The rate near 0°C (Fig. 5) is slow but remains nearly linear for at least 3 days. The rate of tetramerization rises with ribosomal concentration, but gradually reaches a plateau near 15 mg/ml and again decreases. Other experiments in progress show that the rate of tetramerization in the presence of 10 mM $MgCl_2$ is relatively independent of KCl concentration between 0.1 and 0.3 M, and of pH between pH 6.9 and 9.4. Variation of temperature yields a result that is surprising in light of the "hypothermic" nature of the process *in vivo:* the rate *increases* with temperature to temperatures

TABLE 1 *Poly(U) stimulation of polyphenylalanine synthesis*

Ribosomes	With poly(U) (cpm/0.10 mg ribosomes)	Without poly(U) (cpm/0.10 mg ribosomes)
Free ribosomes from normal embryos	2730	75
Polysomes from normal embryos	1540	360
Free ribosomes from cooled embryos	1350	70
Tetramers from cooled embryos	1850	155
No ribosomes	45	40

Poly(U) stimulation of phenylalanine incorporation into trichloroacetic acid-insoluble material in a system similar to that of Arnstein, Cox, and Hunt (7). 0.1 mg of ribosomes was incubated for 30 min at 37°C in the presence of the following components: ATP, 1 mM; GTP, 0.4 mM; phosphoenol pyruvate, 10 mM; pyruvate kinase, 10 μg/ml; $MgCl_2$, 15 mM; KCl, 80 mM; 2-mercaptoethanol, 5 mM; pH 5 enzymes and RNA, 0.25 volume; poly(U), 0.1 mg/ml, when used; and [^{14}C]phenylalanine, 6.6 μM at 95 Ci/mol (this specific activity was further diluted by endogenous phenylalanine in the ribosome and pH 5 fractions). The solution of pH 5 enzymes and tRNA was prepared by adjusting the upper 6 ml of preparative gradients from normal tissue to pH 5.0 with acetic acid and resuspending the precipitate in 1 ml of regular buffer; this opalescent suspension was stored at −70°C and thawed immediately before use. Incorporations were performed in total volumes of 0.4 ml, stopped by the addition of a 100-fold excess of [^{12}C]phenylalanine 30-sec before the addition of an equal volume of 10% trichloroacetic acid; these samples were heated to 90°C for 15 min and filtered onto glass filters for liquid scintillation counting in toluene at 60% efficiency.





(Left) FIG. 5. Tetramer formation *in vitro* by puromycin-treated ribosomes from normal embryos. Ribosomes were incubated at 0°C at various concentrations in normal buffer and analyzed periodically by gradient analysis. Aliquots equivalent to 0.1 mg of ribosomes were removed from each sample, diluted to 0.3 ml in buffer, and heated to 37°C for 5 min before layering on 10–25% sucrose gradients in the Spinco SW-41 rotor. After 60 min of centrifugation at 40,000 rpm, 4°C, absorbance profiles at 254 nm were determined in the ISCO density gradient analyzer and the heavier peaks were confirmed by electron microscopy to consist of typical tetramers. Peak areas were measured by planimetry and the percentage of tetramers/total ribosomes calculated: (*a*) The set of profiles obtained at 101 hr. (*b*) Composite data for the kinetics of tetramerization under these conditions.

(Right) FIG. 6. The presence of nascent polyphenylalanine on tetramers and free ribosomes displayed on sucrose gradients. Purified tetramers were incubated in the poly(U)-stimulated polyphenylalanine-synthesizing system described in Table 1, except that 0.20 mg of tetramers and 0.03 mM [^{14}C]phenylalanine (95 Ci/mol) were used in each of the two 0.4-ml reactions. Both tubes were incubated for 20 min at 37°C, one tube receiving an addition of puromycin to 4 mM after 15 min of incubation. The samples were rapidly cooled to 0°C and subjected to sucrose gradient separation as in Fig. 5. It can be seen that about half of the tetramers have dissociated to free ribosomes during their purification and incubation. Fractions were precipitated with 10% trichloroacetic acid in the presence of added 0.2 mM [^{12}C]phenylalanine and 0.1% bovine serum albumin, hydrolyzed, filtered, and counted as before. *Upper:* sample incubated normally for 20 min. *Lower:* sample receiving puromycin at 15 min of incubation. Absorbance (——). Radioactivity (·–·–·–·).

above 30°C with an approximate Q_{10} of 2 between 0 and 20°C. There is an obvious decrease in light scattering by these suspensions at higher temperatures. This observation, as well as the decrease of rate at very high ribosome concentrations, suggests that nonspecific aggregation may make the availability of free ribosomes a rate-limiting variable for tetramerization *in vitro*.

Protein synthesis

Before structural analysis of these ribosomes was undertaken, it was also important to determine whether they are complete particles capable of engaging in polypeptide synthesis. Therefore, a cell-free system for poly(U)-stimulated polyphenylalanine synthesis was employed for assaying this activity by purified ribosomes, tetramers, and polysomes extracted from cooled or normal embryos. Under conditions where the concentration of ribosomes was rate-limiting the results in Table 1 were obtained (no amino acids were added other than phenylalanine). Activity was essentially linear for more than 30 min and was inhibited by puromycin. If the 19 other amino acids were added (0.02 mM each), the difference of activity for tetramers in the presence and absence of poly(U) was only 2- to 5-fold, rather than 12-fold, and the results were more variable between experiments. It was not possible, therefore, to conclude whether or not the ribosomes of tetramers formed *in vivo* already contained messenger RNA, but the efficient activity in the presence of poly(U) reveals two interesting features of synthesis by reaction mixtures containing tetramers. One was that tetramers showed a degree of activity similar to that of free ribosomes from the same tissues. The other was that the kinetics of synthesis did not display any lag such as one might expect if tetramers must dissociate to free ribosomes before synthesis could begin. Both features suggested that synthesis might be occurring on the intact tetramers themselves.

In order to test this proposal, complete reaction mixtures were incubated for 20 min at 37°C, layered onto sucrose gradients in buffer, and the monomers and tetramers were separated. Fractionation and hot-trichloroacetic acid precipitation yielded the results shown in Fig. 6. In every experiment, there was the same specific activity of polyphenylalanine (per absorbance unit of ribosomes) in the tetramer peaks as in the monomer peaks. The conclusion that polyphenylalanine synthesis was occurring on the ribosomes of intact tetramers was subjected to various controls. First, if the final 5 min of the incubation was performed with 4 mM puromycin added, the hot-trichloroacetic acid-insoluble material was freed from the ribosomes and found at the top of the gradient. Moreover, a treatment with pancreatic ribonuclease found sufficient to disrupt polysomes (0.2 μg/ml at 0°C for 15 min) significantly reduced neither the absorbance of the tetramer peak nor its radioactivity; this obviates the possibility that a coincident band of polysomes on the gradient carry the incorporated phenylalanine. It still remained conceivable, however, that label was entering the tetramers by rapid equilibrium with functional single ribosomes, because half of the ribosomes had become freed from the tetramers during their purification and incubation. But, as expected from earlier kinetic studies of tetramerization, experiments with free ribosomes competent to form tetramers *in vitro* showed no measurable production of tetramers under the conditions of incubation. Nevertheless, there still remained the possibility that a single bond separa-

tion between two ribosomes of the cyclic tetramer permitted one of these ribosomes to become active. It was found, however, that "light tetramers" of 60S subunits produced on a high-salt gradient (0.6 M KCl, 10 mM $MgCl_2$, 10 mM Tris·HCl, pH 7.4) after the reaction appeared in good yield and retained polyphenylalanine, thereby indicating that all four bonds had remained intact. Furthermore, the original observation that the specific labeling of the tetramer peak is equal to that of the monomer peak, and not just one-fourth as great, indicates that transient separations of tetramer bonds are not involved in the synthesis on tetramers. Although the poly(U)-stimulated synthesis of polyphenylalanine by these tetramers is insufficient to demonstrate their ability to mediate the synthesis of normal proteins, these experiments demonstrate their ability to form peptide bonds while constrained in the tetrameric configuration.

CONCLUSIONS

In addition to providing a basis for the growth of larger crystals of the chick-embryo ribosomes *in vitro*, these studies suggest certain features about the crystals. First, the nature of the "light tetramers" indicates that the fundamental structure is a tetramer of large (60S) subunits to which are attached the small (40S) subunits. Secondly, the demonstrated establishment of both tetramer and dimer bonding *in vitro* suggests that there are no easily dissociable factors necessary for growth of the *p4* crystal. Finally, the synthetic studies indicate that the active sites for polypeptide synthesis are not obstructed by tetrameric aggregation. Whether there may be a unique biological function for these aggregates remains unknown, but they appear to constitute a useful system for the structural analysis of functional ribosomes.

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