RIBOSOME CRYSTALLIZATION IN CHICKEN EMBRYOS

II. Conditions for the Formation of Ribosome Tetramers In Vivo

T. MORIMOTO, G. BLOBEL, and D. D. SABATINI

From The Rockefeller University, New York 10021

ABSTRACT

Slow cooling of fertilized chicken eggs permits the elongation and termination of nascent polypeptides in the polysomes but prevents the initiation of new protein chains. This leads to polysome disaggregation during the first 30 min of cooling, and to the formation, of a pool of inactive ribosomes prone to crystallization. After 2 hr these ribosomes began to form tetramers, which do not contain any labeled proteins synthesized during cooling. If protein synthesis is inhibited by cycloheximide, added to eggs before cooling, tetramer formation in the embryos is prevented. Puromycin, on the other hand, leads to polysome disassembly and does not prevent tetramer formation. Rapid cooling of explanted embryos after short incubation at 37°C, with or without cycloheximide, largely prevents polysome disaggregation and the formation of tetramers. On the other hand, the addition of puromycin to explanted embryos promotes tetramer formation after rapid cooling. When cooled eggs are rewarmed, tetramers are disassembled into monomers, even if protein synthesis is inhibited. When those embryos were rapidly recooled tetramers reformed spontaneously from tetramer-derived monomers, even in the presence of cycloheximide. We conclude that the formation of tetramers at low temperature is an inherent property of the normal ribosomes.

INTRODUCTION

In the preceding paper (Morimoto et al., 1971) we have demonstrated that inactive but potentially functional 80S mature ribosomes are the constituents of the crystalline lattices found in hypothermic chicken embryos. We now present a study of the formation of ribosome crystals during cooling, in which we attempt to elucidate the role of protein synthesis in the production of tetramers.

MATERIALS AND METHODS

(a) *Materials*

5-day old fertilized chicken eggs were used, in which, after 24 hr of cooling, ~30% of the ribosomes in the postnuclear supernatant occur as tetramers (Morimoto et al., 1971). The procedure for slow cooling, and the composition of solutions A, B, and C were described previously (Morimoto et al., 1971).

(b) *Amino Acid Incorporation In Vivo*

A small window (~0.5 cm^2) (Rugh, 1962) was cut in the shell of 5-day old fertilized eggs and used to deposit through it onto the chorio-allantoic membrane, without removing the eggs from the incubator, leucine-4,5-^{3}H (SA 56 Ci/mmole, New England Nuclear Corp., Boston, Mass.), antibiotics, or distilled water.[1] After each treatment, the window

[1] In preliminary experiments we found that when the same amount of leucine-^{3}H was given in dif-

was covered with sterilized adhesive tape and the incubation at 39°C was continued, or cooling began immediately by transferring the eggs to a cold room at 4°C.

(c) *Preparation of the Postnuclear Supernatant*

Eggs were removed at various times of incubation or cooling, and opened in a Petri dish at room temperature. The amnion was quickly excised, the embryos were transferred to cold saline, cleaned from any accompanying membranes or yolk, washed twice with solution B, and then homogenized in solution B (0.4 ml per embryo), using 5 strokes of a Potter-Elvehjem homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 600 *g* for 5 min at 4°C and the supernatant (postnuclear supernatant) was collected and used for sucrose density gradient analysis as described in the previous paper (Morimoto et al., 1971).

(d) *Determination of the State of Aggregation of Ribosomes within Embryos*

Portions of the postnuclear supernatant (0.3 ml containing ~15 OD260 units) were analyzed by sucrose density gradient centrifugation as previously described (Morimoto et al., 1971). The distribution of ribosomes as polysomes, monomers and dimers, or tetramers was quantitated by measuring the areas under the corresponding peaks in the sedimentation patterns.

To facilitate the quantitation of tetramers during the early stages of cooling, postnuclear supernatants were treated with RNase (10 μg pancreatic RNase, Worthington Biochemical Corp., Freehold, N. J., in 0.3 ml of postnuclear supernatant containing ~15 OD260 units) for 10 min at 0°C to degrade polysomes into monomers. We have shown that at Mg^{++} concentrations higher than 2 mM, RNase treatment in a solution containing 50 mM triethanolamine-HCl pH 7.5, and 50 mM KCl does not disassemble tetramers (Morimoto et al., 1971).

(e) *Procedures*

The procedures used to measure protein, RNA and radioactivity incorporated into hot and cold acid-insoluble products in the homogenate or in fractions collected from the sucrose density gradients were described previously (Morimoto et al., 1971).

ferent volumes between 50 and 200 μl, the amount incorporated by the embryos during the first hour of incubation was independent of the volume in which the amino acid was administered.

RESULTS

(1) *Changes in the State of Aggregation of Ribosomes during Slow Cooling*

The state of aggregation of ribosomes before and during cooling was determined by sucrose density gradient analysis of postnuclear supernatants, which in all cases contained ~65% of the total RNA in the embryo and are therefore representative of the state of ribosome aggregation (see previous paper, Morimoto et al., 1971). In rapidly homogenized, control embryos (Fig. 1 *a*) 20% of the ribosomes were found as monomers, and more than 70% as polysomes. Fig. 1 *b* shows, however, that postnuclear supernatants from control eggs which received cycloheximide 5 min before homogenization, to inhibit any completion of nascent chains which may occur during cell fractionation, had a larger fraction of polysomes (~90%) and a corresponding decrease in the proportion of monomers. It is likely, therefore, that a pattern such as that in Fig. 1 *b* more truly reflects the distribution of ribosomes within

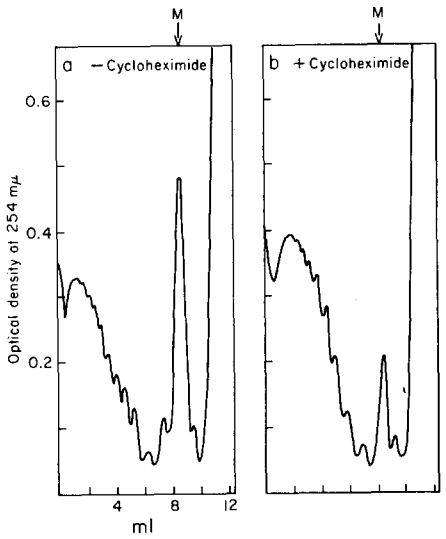

FIGURE 1 State of aggregation of ribosomes in 5-day old embryos. During incubation at 39°, 50 μl of distilled water (*a*) or 50 μl of cycloheximide (5×10^{-4} M) (*b*) were deposited on the chorioallantoic membrane. After 5 min of incubation, two embryos of each treatment were removed and homogenized together in solution B. Portions (~15 OD_{260} units) of postnuclear supernatants were layered on 12.5 ml, 10–40% sucrose density gradients of the same compositions as solution A, which were centrifuged at 40,000 rpm for 100 min at 4°C.

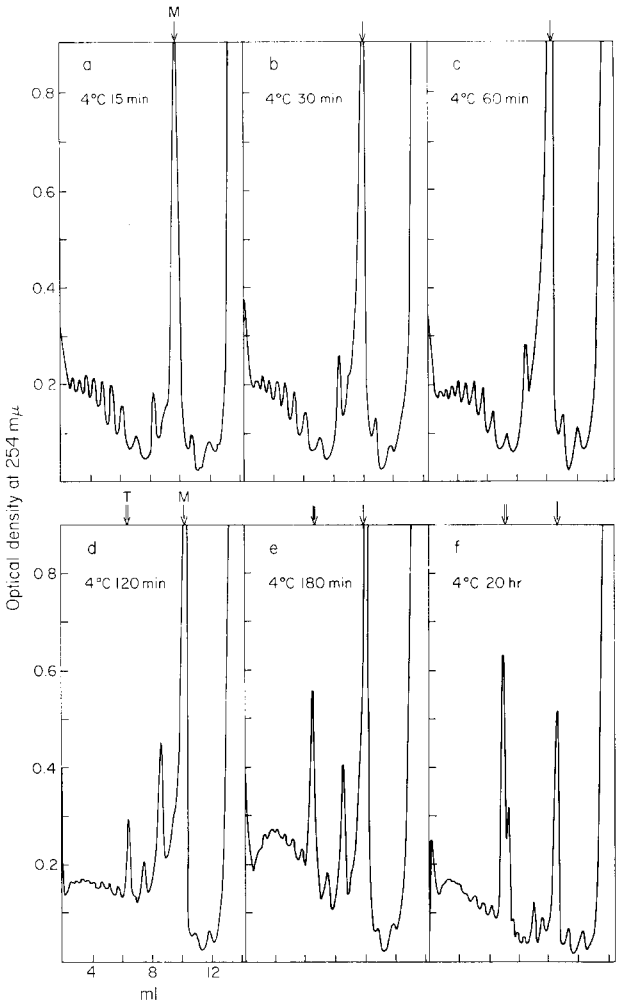

FIGURE 2 Changes induced by cooling in the state of aggregation of ribosomes. 5-day old fertilized eggs were transferred to a cold room at 4°C. At the times indicated, two embryos were removed and postnuclear supernatants were prepared and analyzed as in Fig. 1.

the normal 5 day old embryo than the one in Fig. 1 *a*.- Changes occurring during cooling are illustrated in Figs. 2 and 3, where the relative amount of polysomes and monomers and the appearance of tetramers are expressed as percentages of those in Fig. 1 *b*. The proportion of each class of ribosomes was measured from sedimentation patterns, which like those shown in Fig. 2, were obtained from postnuclear supernatants treated with (not shown here) and without RNase (Fig. 2), to calculate the proportion of monomers. It is clear in Figs. 2 and 3 that the main dissociation of polysomes into monomers occurred during the first 30 min of cooling, after which ~40% of the ribosomes were monomers. Tetramers, on the other hand, appeared only after 2 hr of cooling, and their amount increased during the first 15 hr although the amount of polysomes remained almost constant during this period. The appearance and accumulation of tetramers was accompanied by a decrease in the amount of monomers. Since control experiments using labeled orotic acid (Fig. 6) showed that the synthesis of ribosomes during 24 hr of cooling is negligible, the sequence of events depicted in Fig. 3 strongly suggests that tetramers are formed from monomers which are released from polysomes during the first 30 min of cooling. At this

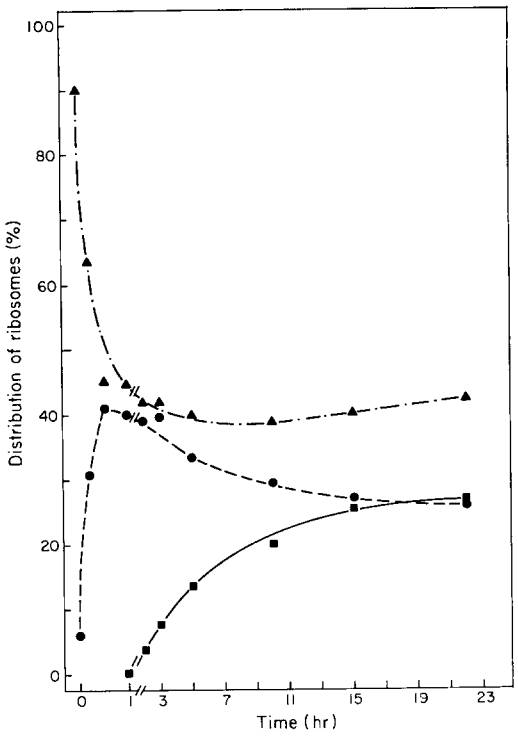

FIGURE 3 Kinetics of ribosome crystallization induced by slow cooling. 5-day old fertilized eggs were cooled for the lengths of time indicated. The proportion of tetramers (■—■—■) was calculated from sedimentation patterns of postnuclear supernatants treated with RNase. Patterns from postnuclear supernatants not treated with RNase (as in Fig. 2) were used to calculate the proportion of total ribosomes present as polysomes (▲···▲···) and monomers (●---●---●).

time, measurements of the temperature within the egg showed that the yolk temperature was ~25°C. As will be shown later, rapid cooling of an explanted embryo by immersion in ice-cold solution C, followed by incubation at 0°C for up to 24 hr, does not result in an extensive production of tetramers. Because of these observations, we designed experiments to test possible correlations between protein synthesis and ribosome crystallization during cooling.

(2) *Protein Synthesis during Slow Cooling*

We first determined the rate of protein synthesis in control and slowly cooled embryos, as well as the effect of antibiotics on this process. The protein content of a 5 day old embryo (initially ~5 mg of protein per embryo) increases twofold over a period of 24 hr of incubation at 39°C. Fig. 4 shows the course of in vivo incorporation of leucine-^{3}H administered to the chorio-allantoic membrane of a control embryo. The rate of protein synthesis can be measured from the slope of leucine-^{3}H incorporation into total protein, which during the first 90 min proceeds almost linearly (Fig. 4). Cycloheximide and puromycin quickly and effectively inhibited in vivo protein synthesis. Slow cooling was also an effective inhibitor of protein synthesis as shown by administering leucine-^{3}H immediately before transferring the eggs to 4°C (not shown). The incorporation of leucine-^{3}H and the increase of total protein in embryos, over 24 hr of cooling, were equivalent to those in control embryos incubated for only ~30 min at 39°C. As expected, protein synthesis during cooling was further inhibited by cycloheximide or puromycin: the amount of leucine-^{3}H incorporated during 24 hr of cooling was less than 10% (with cycloheximide) or 15% (with puromycin) of the amount incorporated by embryos cooled in the egg with leucine-^{3}H but without antibiotics. Results similar to those just described were obtained using explanted embryos incubated in solution C with leucine-^{3}H and/or the antibiotics.

(3) *The Effect of Protein Synthesis Inhibitors on the Formation of Tetramers Induced by Slow Cooling*

Embryos cooled *in ovo* which, before the beginning of slow cooling, received leucine-^{3}H with or without cycloheximide or puromycin were used to prepare postnuclear supernatants which were incubated with or without RNase and then analyzed in sucrose density gradients. The optical density profiles (Fig. 5) showed that after 22 hr of cooling without antibiotics (Fig. 5 *a*, *b*) ~30% of the ribosomes were present as tetramers, ~40% as polysomes, and the rest as free monomers. The distribution of cold and hot acid–insoluble leucine-^{3}H radioactivity demonstrated that tetramers *do not contain* nascent polypeptide chains or amino acyl tRNA, labeled during cooling; most of the incorporated label was in polysomes (Fig. 5 *a*) or remained near the top of the gradients. As expected, RNase treatment did not affect the tetramer peak, but converted all polysomes into monomers, which retained the labeled nascent polypeptides (Fig. 5 *b*).

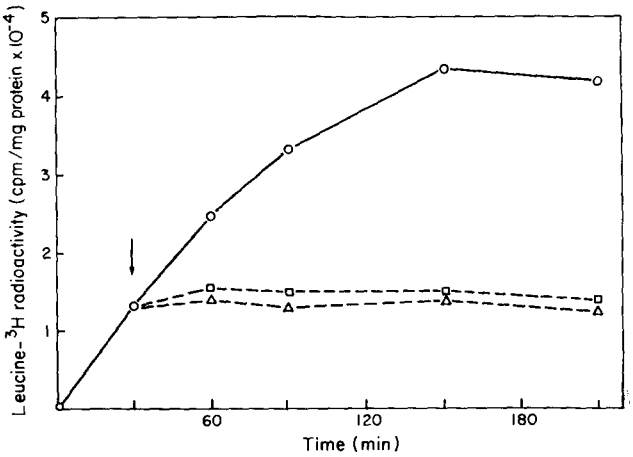

FIGURE 4 Effect of cycloheximide and puromycin on the in vivo incorporation of leucine-^{3}H. Leucine-^{3}H (10 μCi in 50 μl) was administered to 5-day old embryos through a window made in the egg shell 1 hr before. After 30 min, 50 μl of distilled water (○—○—○) or 50 μl of 5×10^{-3} M cycloheximide (△---△---△) or 50 μl of 10^{-2} M puromycin (□---□---□) were similarly administered. At the times indicated, two embryos of each treatment were removed from the eggs, transferred into ice-cold saline, washed, and homogenized in solution B. Protein in the homogenate and radioactivity incorporated into total protein were measured as described in Materials and Methods (section e).

The addition of antibiotics had striking and specific effects on the formation of tetramers by slow cooling: (1) Cycloheximide administered before cooling almost completely suppressed the formation of tetramers (Figs. 5 *c*, *d*). As shown for other systems (Colombo et al., 1965), the polysomes were preserved by cycloheximide treatment; they contained a part of the small amount of leucine-^{3}H incorporated, the rest being present in soluble proteins (Fig. 5 *c*). No peak corresponding to the RNase-resistant tetramers was observed when these polysomes were converted into monomers by RNase (Fig. 5 *d*). (2) Puromycin administered before cooling did not prevent the formation of tetramers during cooling (Figs. 5 *e*, *f*), although, as we indicated before, protein synthesis during cooling was almost as effectively inhibited by puromycin as by cycloheximide. The amount of polysomes in embryos from eggs which received puromycin was small, and the optical density profile was similar to that of untreated cooled eggs. The distribution of radioactivity clearly showed that the polysomes from puromycin-treated embryos do not contain nascent polypeptides (Fig. 5 *e*). All labeled nascent polypeptides in this case were soluble, and remained at the top of the gradients. As in the previous cases, RNase treatment converted polysomes into monomers, but did not affect the amount of tetramers or the distribution of the label (Fig. 5 *f*).

The observation that tetramers do not contain nascent polypeptides labeled during cooling, together with the kinetics of tetramer formation (Fig. 3) and the effect of cycloheximide, suggests that the completion and release of nascent chains during cooling is necessary for the formation of tetramers. Moreover, the result with puromycin suggests that protein synthesis is needed only to terminate nascent chains and to release monomers from polysomes, since ribosomes stripped of nascent chains by puromycin and artificially released from polysomes can also form tetramers in the cold.

Cold and hot acid-insoluble radioactivities had a similar distribution in each of the gradients shown in Fig. 5. Cold acid-insoluble radioactivity in polysomes and monosomes was higher than hot acid-insoluble radioactivity, probably because some aminoacyl- and short peptidyl-tRNA molecules were solubilized by hot acid hydrolysis.

(4) *RNA Synthesis during Cooling*

Attempts were made to measure RNA synthesis during cooling by following the incorporation into RNA of orotic acid hydrate-5-^{3}H (2810 mCi/mmole, New England Nuclear

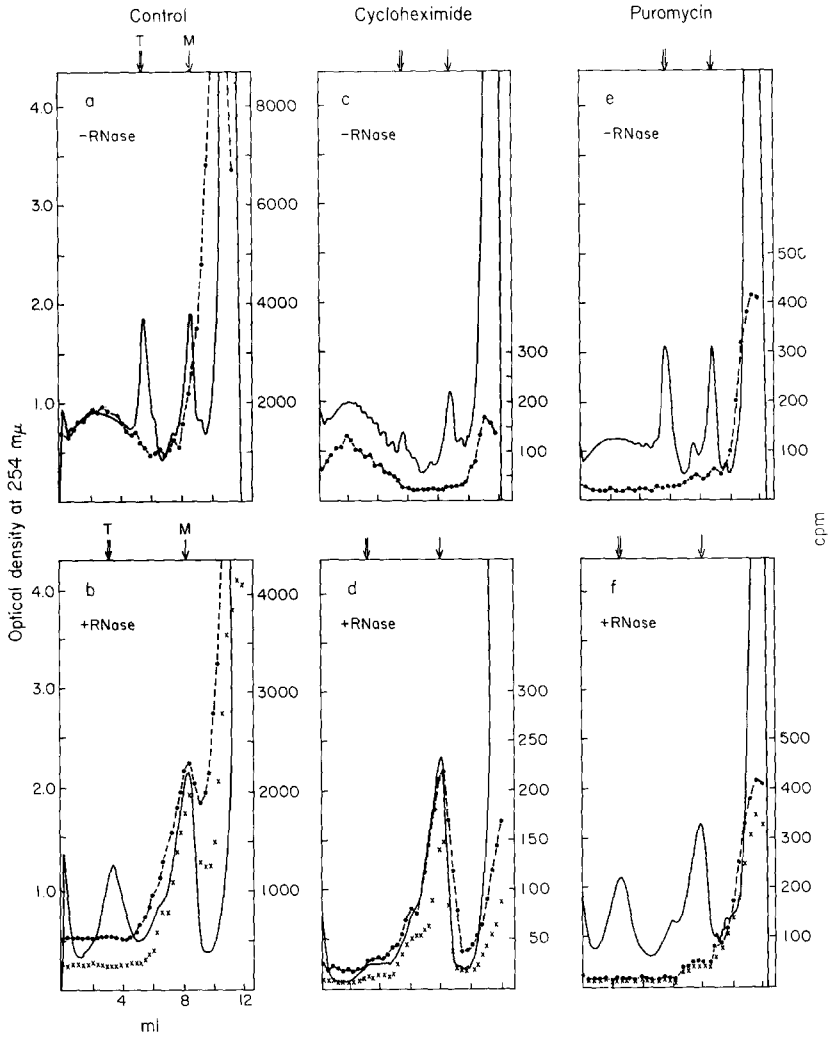

FIGURE 5 Effect of protein synthesis inhibitors on tetramer formation. 5-day old fertilized eggs received, through a window in the egg shell (Materials and Methods, section b), 60 μl of a solution containing 10 μCi of leucine-^{3}H alone (*a*, *b*) or 10 μCi leucine-^{3}H with 2.5×10^{-7} moles cycloheximide (*c*, *d*) or 10 μCi leucine-^{3}H with 5×10^{-7} moles puromycin (*e*, *f*). After slow cooling for 22 hr, four embryos of each group were homogenized together in 1.6 ml of solution B. From each homogenate two 0.2 ml portions were removed to measure protein and hot and cold acid-insoluble radioactivities. Postnuclear supernatants were prepared, portions (0.8 ml each containing 40 OD_{260} units) of which were incubated with (*b*, *d*, *f*) or without (*a*, *c*, *e*) RNase, and layered onto 12 ml, 10–40% (*a*, *c*, *e*) or 7.5–20% (*b*, *d*, *f*) sucrose density gradients prepared in solution A. Gradients were centrifuged at 40,000 rpm for 100 min at 4°C. Hot x x x) and cold (●---●---●) acid-insoluble radioactivities throughout the gradients were measured as indicated in Materials and Methods, section e. ——, optical density at 254 mμ.

Corp.) administered (20 μCi in 50 μl) to the eggs before transfer to 4°C. Over a period of 12 hr, embryos cooled in the egg incorporated only ~10% of the value incorporated by controls. Figs. 6 *a*, *b* correspond to sucrose density gradient patterns of postnuclear supernatants and show the distribution in ribosomes from cooled (*b*) and control (*a*) embryonated eggs. From the radioactivity curve it is clear that there was negligible incorporation into tetramer-associated RNA's.

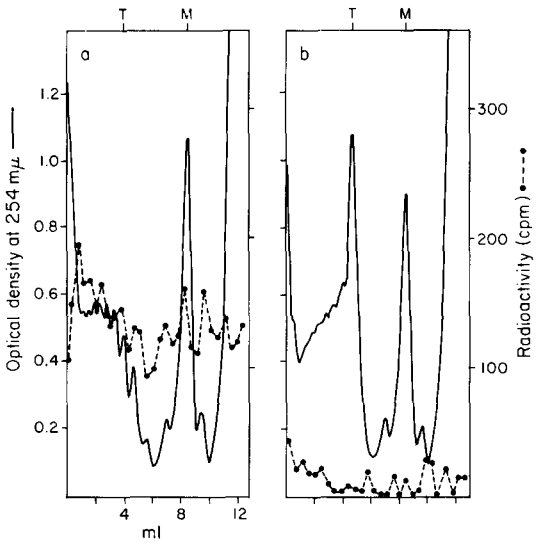

FIGURE 6 Incorporation of orotic acid-^{3}H into RNA during cooling. After receiving 50 μl of orotic-^{3}H acid (20 μCi) in the chorio-allantoic membrane, eggs were reincubated at 39°C (*a*), or immediately transferred to 4°C (*b*). After 12 hr, pairs of embryos for each group were removed and homogenized together. Portions (0.4 ml containing 30 OD_{260} units) were analyzed in 12.5 ml, 10–40%, sucrose density gradients of the same ionic composition as solution A, which were centrifuged for 2 hr at 40,000 rpm. 0.4 ml fractions from the effluent of the optical density monitor were precipitated with cold 5% trichloroacetic acid, collected on filter paper discs, washed and extracted with alcohol and alcohol:ether (1:1). The radioactivity in the discs was measured as previously described (Morimoto et al. 1971). ——, optical density at 254 mμ; ●---------●, radioactivity (cpm).

(5) *Effect of Metabolic Inhibitors on Tetramer Formation*

The administration of 50 μl of a solution containing dinitrophenol (1 μmole), or sodium fluoride (10 μmoles), or sodium azide (1 μmole) to the chorio-allantoic membrane immediately before cooling the embryonated eggs, did not prevent tetramer formation during cooling, although protein synthesis was reduced to 5–20% of that in controls. Sodium cyanide (1 μmole) or monoiodoacetic acid (1 μmole) also inhibited protein synthesis, during cooling, to levels which were 10–30% of those in controls, but these two inhibitors effectively prevented tetramer formation and maintained the relative concentration of polysomes during cooling.

(6) *Tetramer Formation in Rapidly Cooled Embryos*

Since it is technically difficult to cool whole eggs rapidly, embryos were explanted and cooled by immersion into ice-cold solution C. We first checked whether protein synthesis (measured by leucine-^{3}H incorporation) proceeds in 5-day old explanted embryos incubated at 37°C and whether it can be inhibited by antibiotics and by rapid cooling. The incorporation of leucine-^{3}H into total embryonic protein (measured in homogenates) proceeded at a linear rate for ~2 hr and was effectively inhibited (over 90%) by puromycin (5×10^{-4} M) or cycloheximide (2.5×10^{-4} M) each added together with the amino acid or after 1 hr. Embryos incubated for 1 hr at 37°C with leucine-^{3}H and then rapidly cooled by immersing the tubes in an ice bath showed over 95% inhibition of protein synthesis during the next hour. These results indicated that rapid cooling was considerably more effective than slow cooling in inhibiting protein synthesis in the embryos. The effect of rapid cooling and/or antibiotics on the state of aggregation of ribosomes in the postnuclear supernatant of the explanted embryos was then studied by density gradient centrifugation (Fig. 7).

The results showed that: (1) in explanted embryos incubated at 37°C for 8 min without antibiotics (Fig. 7 *a*), or with cycloheximide (Fig. 7 *d*), most ribosomes exist as polysomes and monomers. Rapid cooling of these embryos, followed by incubation at 0°C for 20 hr, results in the production of a small but detectable amount of tetramers (Figs. 7 *b*, *e*). This is especially clear in gradients from samples treated with RNase to eliminate polysomes (Figs. 7 *c*, *f*). Tetramer formation is, however, more than 10 times less efficient than in slowly cooled embryonated eggs. (2) Puromycin leads to an increase in the amount of monomers in explanted embryos, mainly by disaggregation of large polysomes (Fig. 7 *g*). Strikingly, rapid cooling after incubation with puromycin, followed by 20 hr of incubation at 0°C, resulted in the production of a large amount of tetramers (Figs. 7 *h*, *i*), similar to that obtained after slow cooling of intact or puromycin-treated eggs (Figs. 5 *a*, *b*, *e*, *f*). The tetramers produced by rapid cooling in the presence of puromycin show the usual resistance to RNase (Fig. 7 *i*).

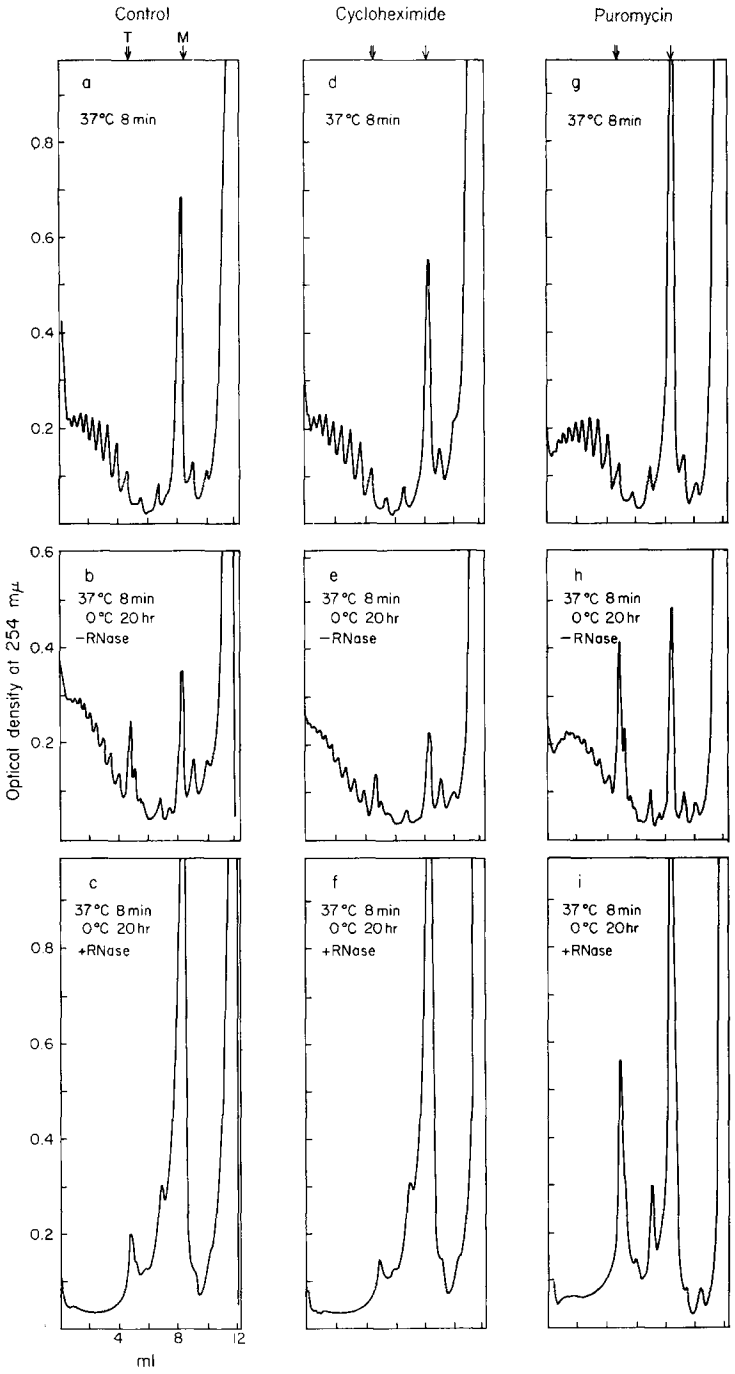

FIGURE 7 Tetramer formation in rapidly cooled embryos treated with cycloheximide or puromycin. Embryos were removed from 5-day old eggs, as described in Materials and Methods, section c, washed with solution C, and transferred (two embryos per tube) to 1 ml of cold solution C containing either 10 μCi of leucine-^{3}H (*a, b, c*); 10 μCi of leucine-^{3}H and 2.5×10^{-7} moles cycloheximide (*d, e, f*) or 10 μCi of leucine-^{3}H and 5×10^{-7} moles puromycin (*g, h, i*). After 20 min at 0°C, the tubes were transferred to a water bath (37°C), incubated for 8 min, and rapidly cooled by immersion into an ice bath, where they were kept for 20 hr. At the end of incubation at 37°C (*a, d, g*) and the end of cooling (*b, c, e, f, h, i*), four embryos of each group were homogenized in solution B. From each homogenate, 0.2 ml portions were used to measure total protein and leucine-^{3}H incorporation (see text, Results, section 6). From the rest, postnuclear supernatants were prepared, portions of which were incubated with (*c, f, i*) or without (*a, b, d, e, g, h*) RNase. The samples were finally analyzed by density gradient as described for Fig. 5, except that they were centrifuged for 120 min.

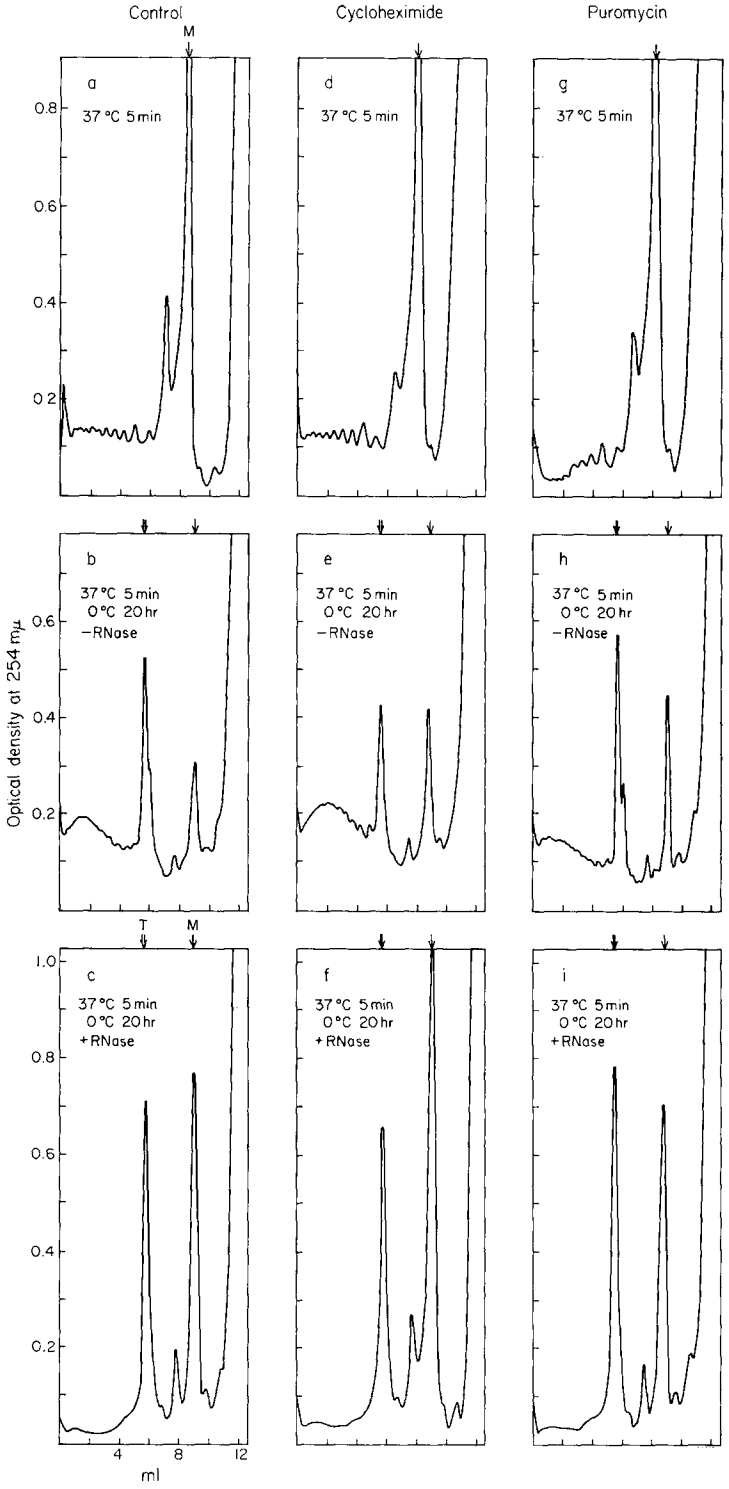

FIGURE 8 Dissociation and reformation of tetramers in the presence of cycloheximide or puromycin The embryos used in this experiment were removed from 5-day old eggs, slowly cooled for 22 hr, and had a large fraction of their ribosomes in tetramers (see Fig. 5). After washing twice in cold solution C, the embryos were transferred (2 embryos per tube) to 1 ml of cold solution C with 10 μl of leucine-^{3}H (*a*, *b*, *c*), 10 μCi of leucine-^{3}H with 2.5×10^{-4} M cycloheximide (*d*, *e*, *f*) or 10 μCi of leucine-^{3}H with 5×10^{-4} M puromycin (*g*, *h*, *i*). After 20 min at 0°C, the tubes were transferred to a water bath (37°C), incubated for 5 min, and rapidly cooled by immersion into an ice bath, where they were kept for 20 hr. At the end of incubation at 37°C (*a*, *d*, *g*) and the end of recooling at 0°C (*v*, *c*, *e*, *f*, *h*, *i*), four embryos for each group were homogenized in solution B. Total protein and the incorporation of leucine-^{3}H were measured in two 0.2 ml portions of the homogenate, and the results are given in the text (Results, section 7). Postnuclear supernatants were prepared and split into portions which were incubated with (*c*, *f*, *i*) or without (*a*, *b*, *d*, *e*, *g*, *h*) RNase and analyzed as described in the legend to Fig. 7.

(7) *Dissociation and Reformation of Tetramers in the Presence of Protein Synthesis Inhibitors*

It has been shown by electron microscopy (Byers, 1966) that intracellular ribosome crystals produced by hypothermic treatment of fertilized eggs disappear upon reincubation of the eggs at warm temperatures. Hence, the following experiments were carried out to see whether rewarming of the embryo also leads to the dissociation of the tetramers and whether protein synthesis is necessary for the breakdown of the ribosome crystals.

5-day old embryos, removed from eggs kept for 24 hr at 4°C, were transferred to tubes containing leucine-^{3}H in solution C, and incubated at 37°C, with or without the addition of cycloheximide or puromycin. During the first 30 min of incubation at 37°C, the rate of protein synthesis (measured in homogenates) in the rewarmed embryos was slow (less than one-half of normal rate), but after this time protein synthesis proceeded normally, at the same rate as in control embryos. Cycloheximide or puromycin given before rewarming effectively inhibited (over 90%) protein synthesis measured during the first 2 hr of rewarming. Sedimentation analysis of the postnuclear supernatants from precooled embryos homogenized immediately before incubation at 37°C gave results similar to those shown in Fig. 5 *a*, demonstrating that a large proportion (~30%) of their ribosomes were in tetramers. Similar analysis after 5 min of incubation at 37°C (Figs. 8 *a*, *d*, *g*) showed that this short rewarming was sufficient in all cases (whether or not cycloheximide or puromycin was present) to produce the complete conversion of tetramers into monomers. However, some of the monomers produced during incubation at 37°C arose from polysomes since the amount of polysomes in samples from embryos rewarmed for only 5 min was smaller than before incubation at 37°C (compare Figs. 8 *a*, *d*, *g* with Fig. 5 *a*). Although neither cycloheximide nor puromycin affected the conversion of tetramers into monomers, it may be worth noting that incubation with puromycin led to a more pronounced reduction in the amount of polysomes. From these observations we concluded that protein synthesis was not required to disassemble tetramers in vivo, and that incubation at 37°C was sufficient.

The following experiments were performed to determine whether tetramers could be reassembled from their products of dissociation after rapidly recooling rewarmed eggs, and whether protein synthesis is necessary for this process. It should be recalled that we have shown that (1) rapid cooling of an embryo *does not* lead to an extensive formation of tetramers from polysomes, and that (2) protein synthesis is needed for the formation of tetramers by slow cooling in embryonated eggs previously incubated at 37°C.

Embryos explanted from hypothermic eggs were incubated at 37°C for 5 min in solution C with or without cycloheximide or puromycin to disassemble the tetramers, then rapidly recooled by immersing the tubes in an ice bath and kept at 0°C for 20 hr. The sedimentation patterns from the postnuclear supernatants incubated with (Figs. 8 *c*, *f*, *i*) or without RNase (Figs. 8 *b*, *e*, *h*) showed that the sequence slow cooling–short warming–rapid recooling led to the formation of tetramers and to a decrease in the amount of monomers, whether or not cycloheximide or puromycin was present. Thus we concluded that protein synthesis was not needed to reform tetramers from tetramer-derived monomers, and that crystallization occurs spontaneously at low temperatures if monomers are available.

DISCUSSION

In attempting to elucidate the conditions under which tetrameric units and crystalline sheets of ribosomes are formed during the cooling of chick embryos, we first considered the possibility that a species of mRNA is synthesized during cooling, which leads to the formation of a special class of polysome or ribosome aggregates. The results presented in the preceding paper (Morimoto et al., 1971) concerning the resistance of tetramers to RNase, their disassociation at high ionic strengths without the need of puromycin, and the results on the incorporation of orotic acid-^{3}H presented here, all indicate that mRNA is absent from the tetramers.

We also investigated the possibility that protein products or nascent polypeptides made in the embryo during the period of cooling are necessary components of the ribosome tetramers. This might be expected since tetramers within chicken embryos appear only after the first 2 hr of cooling at 4°C, a period during which protein synthesis in the embryo still proceeds at a slow rate. We found, however, that proteins labeled during cooling, as well as labeled leucyl-tRNA, are absent from tetramers.

A kinetic study of the changes in ribosome distribution during cooling revealed the formation of a pool of monomers derived from preexisting polysomes during the early stages of cooling. Since the appearance of tetramers occurs later concomitantly with a decrease in the size of the monomer pool, and since the synthesis of new ribosomes during cooling is negligible, these observations suggest that tetramers are formed from monomeric ribosomes released from polysomes. The release follows the completion of the synthesis and the natural discharge of nascent chains as indicated by the absence of nascent polypeptide chains and mRNA in tetramers.

Additional support for the conclusion that tetramer formation requires ribosomes released from polysomes through natural chain termination, was obtained from the following observations: (1) preservation of polysomes through inhibition of protein synthesis—either by rapid cooling of the embryos or by a slow cooling of the eggs in the presence of cycloheximide—almost completely suppressed the formation of the pool of monomers and the subsequent appearance of tetramers. (2) Inhibition of protein synthesis by puromycin, which releases monomers from polysomes by the artificial termination of nascent polypeptide chains, does not prevent tetramer formation either by rapid or slow cooling. (3) Within the embryos, tetramers can be reformed from tetramer-derived monomers by rapid cooling of precooled embryos which, after treatment with cycloheximide or puromycin, were warmed up to disassemble the tetramers. These observations demonstrate that cycloheximide inhibits tetramer formation not because it interferes with the aggregation of ribosomes in tetramers, but because it acts at a previous step which regulates the availability of monomers. Under normal concentrations, cycloheximide is a more effective inhibitor of elongation than of initiation (Lin et al., 1966; Baliga et al., 1969), acting on the translocation step which follows peptide bond formation (Sutter and Moldave, 1966; Munro et al., 1968). By this mechanism cycloheximide inhibits the release of monomers from polysomes which normally occurs during slow cooling, and thereby prevents tetramer formation.

The apparently anomalous effect of puromycin, which does not prevent tetramer formation but does inhibit protein synthesis, is in line with the process proposed, since termination of protein synthesis by puromycin also leads to the formation of a pool of monomers free of nascent chains, which is available for crystallization.

The disassembly of polysomes normally occurring during cooling cannot be attributed to the action of an endogenous RNase, because polysomes were well preserved in embryonated eggs treated with cycloheximide and stored up to 24 hr at 4°C. Moreover, some polysomes always remained apparently unaffected and retained their nascent chains even in eggs which were cooled slowly, and received no cycloheximide. One may also note that an endogenous RNase could not be expected to produce a loss of nascent polypeptides from ribosomes involved in crystallization.

Our observations indicate that, during cooling, elongation and termination of nascent polypeptide chains proceed at a slow rate, even though other metabolic processes may be severely inhibited. We demonstrated that sodium fluoride, sodium azide, and dinitrophenol did not interfere with tetramer formation during cooling, but sodium azide and monoiodoacetate prevented it almost completely Although all these substances were effective in inhibiting protein synthesis, we would suggest on the basis of these observations that they affect differentially the processes of elongation and initiation. An effect of sodium fluoride on initiation has been previously demonstrated (Marks et al., 1963; Lin et al., 1966).

The results of the labeling experiments and the changes in the state of ribosome aggregation just discussed suggest that initiation of new protein chains is differentially suppressed by cooling, while the process of chain elongation and termination continues. In fact, a selective effect of low temperature in inhibiting initiation of protein synthesis has been previously reported in *Escherichia coli* (Das and Goldstein, 1968; Friedman et al., 1969). The possibility should be considered that in our experiments initiation is inhibited because at low temperature ribosomal subunits preferentially associate to form monomers or tetramers and are unavailable to form initiation complexes. It is interesting to note that relatively rapid cooling produces crystals of ribosomes in mitotic cells but not in interphase cells (Byers, 1967). This observation is satisfactorily explained by the mechanism of crystallization proposed by us since it has recently been shown (Fan and Penman, 1970) that initiation of protein synthesis is selectively inhibited in mitotic cells. Thus, a pool of free inactive ribo-

somes is probably formed during mitosis, which is available for crystallization by rapid cooling.

Our description of the sequence of events during cooling is based on analyses of postnuclear supernatants. It is possible, however, that within chicken embryo cells ribosome monomers are not produced directly from polysomes, but that instead ribosomal subunits are released and act as an intermediate between polysomes and tetramers. As suggested for *E. coli* (Phillips et al., 1969), monomers could be formed during homogenization due to the ionic composition of the medium.

Since crystalline sheets of ribosomes are broken down into tetramers during homogenization, it cannot be ascertained whether the formation of tetramers occurs before or at the same time as the formation of sheets. Electron microscope observations did not resolve the question, but showed that isolated tetramers frequently coexist with large sheets of ribosomes in cooled embryonic cells. In fact, such isolated tetramers are predominant in later stages of development, and in chicken tissues cooled during the first week after hatching[2].

The extent to which ribosomes can be involved in crystal formation by cooling at different embryonic ages may depend on factors other than basic structural changes in the ribosomes. The relative abundance of ribosomes, their involvement in protein synthesis, and the sensitivity to cooling of the different steps of protein synthesis—all may affect the availability of ribosomes susceptible to crystallization by cooling. But the actual processes of tetramerization, or of formation of larger aggregates, are likely to be affected by conditions in the intracellular environment which vary with the type of tissue and change with the stage of development.

The observations previously discussed indicate that tetramers are formed from ribosomes not programmed for protein synthesis, which are also free of protein products synthesized immediately before crystallization or during cooling. Crystallization may therefore serve as one means of storing unprogrammed but functionally viable ribosomes in hypothermic eggs. Because we have studied only tetramers, we can not conclude that other cell proteins, present in the embryo as soluble chains completed before cooling, are not required to form large crystals from tetramers. We are now investigating the requirements for the formation of ribosome crystals in vitro and are hoping to obtain conditions for the formation of large crystals suitable for structural studies.

[2] Nonomura, Y., and D. Sabatini. Manuscript in preparation.

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