

RIBOSOME CRYSTALLIZATION IN CHICKEN EMBRYOS

I. Isolation, Characterization, and In Vitro Activity of Ribosome Tetramers

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ABSTRACT

Isolated tetrameric particles (166S) derived from the crystalline lattices known to appear in hypothermic chicken embryos consist of mature 80S ribosomes which contain all species of ribosomal RNA and a complete set of ribosomal proteins.

Ribosome tetramers are not a special type of polysomes since in solutions of high ionic strengths (500 mM KCl and 50 mM triethanolamine-HCl buffer) containing 5 mM MgCl₂ they dissociate into 40S and 60S ribosomal subunits, without the need of puromycin, and at a concentration of Mg⁺⁺ higher than 3 mM they are not disassembled by mild RNase treatment. Tetramers spontaneously disassemble into 80S monomers when the Mg⁺⁺ concentration is lowered to 1 mM at relatively low ionic strength. Tetramers failed to couple in vitro puromycin-³H into an acid-insoluble product, indicating the lack of nascent polypeptide chains. Although tetramers have no endogenous messenger RNA activity, they can be programmed in vitro with polyuridylic acid (poly U) to synthesize polyphenylalanine. All ribosomes within a tetramer can accept poly U, without the need of disassembly of the tetramers into monomers or subunits.

INTRODUCTION

Electron microscope observations (Byers, 1966, 1967) have indicated that, upon cooling fertilized chicken eggs, ribosomes within the embryos form crystalline sheets, the basic unit of which is a tetrameric aggregate. So far, the identification of the aggregated particles as ribosomes is based only on size and staining properties in sections (Byers, 1966, 1967; Maraldi and Barbieri, 1969; Barbieri et al., 1970), and their characterization is limited to data on the sedimentation coefficient of the isolated tetramers and their insensitivity to RNase (Humphreys et al. 1964; Carey, 1970). Assuming that tetramers are formed of ribosomes, it becomes of interest to know what their position in the ribosomal biogenetic and functional cycle is and what the conditions of their formation in vivo are. The studies to be reported in this and

the following paper concern these questions, the answers to which can facilitate the use of the crystals for studies on ribosome organization.

MATERIALS AND METHODS

(a) Media

Solution A, 0.005 M MgCl₂-0.050 M KCl-0.050 M tris(hydroxymethyl)aminomethane (Tris)¹-HCl buf-

¹ Abbreviations: ATP, adenosine triphosphate; EDTA, ethylenediaminetetraacetate; GTP, guanosine triphosphate; HSB, 0.5 M KCl-0.05 M Tris-HCl (pH 7.5)-0.005 M MgCl₂; poly U, polyuridylic acid; TCA, trichloroacetic acid; TEA buffer, triethanolamine-HCl buffer; Tris, tris(hydroxymethyl)aminomethane.

fer (pH 7.5). Solution B, 0.25 M sucrose-0.010 M MgCl_2 -0.050 M KCl-0.050 M Tris-HCl buffer (pH 7.5). Solution C, Krebs-Ringer bicarbonate (Krebs, 1950) equilibrated with a 95% O_2 + 5% CO_2 mixture and supplemented with 19 amino acids except leucine (Eagle, 1959). TEA buffer, triethanolamine-HCl buffer (pH 7.5).

(b) Materials

3-day old fertilized eggs of White Leghorn hens obtained from a commercial farm (Shamrock Farm, N.J.) were incubated at 39°C, with one daily rotation.

(c) Slow Cooling

Eggs were directly transferred to and kept (for up to 24 hr) in a cold room at 4°C. A small thermometer inserted into eggs showed that the yolk temperature reached 4°C after 4 hr of cooling.

(d) Preparation of Ribosomes from 9- to 12-Day Old Chick Embryos

Control or cooled eggs were opened in a Petri dish at room temperature. After excising the amnion, the embryos were transferred to a beaker, washed with cold solution B, and weighed. Usually 15-20 embryos were homogenized at 4°C in one volume of solution B, using five strokes of a motor driven Potter-Elvehjem homogenizer. The homogenate was centrifuged at 600 g for 5 min at 4°C in a model UV International centrifuge (International Equipment Company, Needham Heights, Mass.) to sediment nuclei and unbroken cells, and the supernatant was collected and set aside. The pellet was washed with half the original volume of solution B by rehomogenization and centrifugation under the same conditions. From the combined postnuclear supernatants, mitochondria and large particles were removed as a mitochondrial fraction by centrifugation for 10 min at 15,000 g in the No. 30 rotor of a Spinco L centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The mitochondrial pellet was washed once in solution B, after which the washings and the original supernatant were combined to obtain a postmitochondrial fraction. Ribosomes were obtained from 10-ml portions of this fraction, which were loaded onto discontinuous sucrose density gradients made up of 10 ml of 2.0 M sucrose and 5 ml of 1.5 M sucrose, both with the same ionic composition as solution A. After centrifugation at 30,000 rpm for 24 hr at 2°C in the Spinco No. 30 rotor, the liquid content of the tubes was removed with a syringe and the surface of the colorless and transparent ribosome pellets was rinsed with a small amount of solution A, which was quickly decanted. The yield of ribosomes thus obtained, calculated by

using a value of 135 for the $E_{260}^{1\%}$ (Tashiro and Siekevitz, 1965), was similar for control and cooled eggs (1.3 mg/g of embryo). The ratio of the absorbances at 280 and 260 m μ for ribosomes from both sources was 0.56 ± 0.02 .

(e) Isolation and Purification of Tetramers from Crude Ribosomes

To prepare tetramers, ribosomes from cooled eggs were suspended in a small volume of solution A, using a glass homogenizer operated by hand. After centrifugation (5000 rpm for 10 min in the Spinco No. 40 rotor) to remove large aggregates, the suspension was diluted with solution A to ~ 4 mg/ml. Portions (1.5 ml) were layered onto 7.5-20% linear sucrose density gradients (32 ml in tubes of the SW25 Spinco rotor) of the same ionic composition as solution A. The gradients were centrifuged at 25,000 rpm for 3 hr, after which the optical density throughout the gradients was read at 254 m μ with an ISCO model D fractionator and analyzer (Instrumentation Specialities Co., Inc., Lincoln, Neb.). The effluent corresponding to the prominent peak in the sedimentation profile at the bottom third of the gradient ($\sim 166S$) was collected into three fractions, one for each limb and another for the center of the peak (total ~ 5 ml). These fractions (containing $\sim 13\%$ of the layered amount of ribosomes) were dialyzed against solution A (3-5 hr at 0°C) to remove sucrose. When further purification was needed the middle fraction was reprocessed by sucrose density gradient centrifugation. To concentrate the tetramers, as for the *in vitro* amino acid incorporation experiments (Materials and Methods, section j), the dialysate of the middle fraction was layered on top of 4 ml of 2.0 M sucrose in solution A and centrifuged for 24 hr 40,000 rpm in the Spinco No. 40 rotor. After this step, the resuspended pellet had a ratio of absorbances at 280 m μ and 260 m μ of 0.545 ± 0.005 , and subsequent sedimentation analysis showed that more than 90% of its material consisted of 166S particles.

Sucrose density gradients similar to those described for tetramers were also used to prepare normal monomers from polysomes of uncooled eggs.

(f) RNA Extraction and Analysis

RNA from polysomes and tetramers was extracted with diethyl pyrocarbonate and sodium dodecyl sulfate (Solymosy et al., 1968). For sedimentation analysis the RNA was dissolved in 0.1 M NaCl-0.05 M acetate buffer (pH 5.2), precipitated three times with cold (-15°C) 70% ethanol, and redissolved and dialyzed against the same salt-buffer solution for 3 hr at 0°C.

Total RNA after alcohol precipitation was ana-

lyzed by gel electrophoresis, using 2.5% acrylamide-0.5% agarose composite gels (Peacock and Dingman, 1967). The gels received 15 μ g of RNA in each sample, were run at 200 v for 100 min at 20°C, and were stained with methylene blue. To analyze the material released from tetramers and polysomes treated with ethylenediaminetetraacetate (EDTA), whole samples (170 μ g each) of EDTA-treated ribosomes (5 μ g EDTA/mg ribosomes) were loaded onto 5% acrylamide gels. Electrophoresis at 70 v was run for 3 hr at 15°C. After staining with Stains-all (Eastman Organic Chemicals, Eastman Kodak Co., Rochester, N.Y.) (Dahlberg et al., 1969), the optical density at 570 m μ , throughout the gels, was traced with a Gilford spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio), model 240 equipped with a Gilford linear transporter model 2410.

(g) *In Vitro* Coupling of Puromycin to Nascent Polypeptides

The reaction mixture for puromycin-polypeptide coupling (Blobel and Sabatini, 1971) contained in 210 μ l: 10 μ l of *O*-methyl-puromycin-³H (1 μ Ci) (1.11 Ci/mmol, New England Nuclear Corp., Boston, Mass.), 100 μ l of ribosomes in solution A (polysomes: 750 μ g/ml and 1500 μ g/ml, tetramers: 700 μ g/ml), and 100 μ l of a compensating buffer to adjust the final concentrations to 25 mM Mg⁺⁺-50 mM Tris-HCl (pH 7.5) and various concentrations of KCl. The reaction was carried out at 0°C and started by addition of puromycin-³H. After 2 hr, two 100 μ l portions were pipetted onto Whatman 3 MM filter discs. The puromycin radioactivity incorporated into the hot acid-insoluble residue was measured as described in section *i* of Materials and Methods.

(h) *Sucrose Density Gradient Analysis*

Linear sucrose density gradients (Britten and Roberts, 1960) (12.5 ml) were prepared in tubes of the SB283 rotor of the International B.60 centrifuge (International Equipment Company). Centrifugation, without braking, was carried out at 2°C and at 40,000 rpm for the times indicated in the figure legends (usually for 100-150 min). The optical density throughout the gradients was read with the ISCO monitor (see Materials and Methods section *e*), the effluent of which was collected in 30 fractions (\sim 0.4 ml each) to measure the distribution of hot and cold acid-insoluble radioactivities. In all figures the direction of sedimentation is from right to left.

(i) *Measurement of Radioactivity in the Fractions from Density Gradients*

Upon addition of an equal volume of cold 10% trichloroacetic acid (TCA), the fractions were kept

overnight at 4°C. The TCA precipitates were trapped by filtration on Whatman 3 MM filter paper discs, which were washed under suction with cold 5% TCA, ethanol, ethanol:ether (1:1) and air dried. The cold TCA-insoluble radioactivity in the discs was measured by liquid scintillation counting in 10 ml of Liquifluor (New England Nuclear Corp.). To measure the hot acid-insoluble radioactivity, the discs were removed from the scintillation vials, washed twice with toluene, air dried, and replaced in cold 5% TCA. After 15 min, nucleic acids were hydrolyzed, and the discs were processed according to Mans and Novelli (1960), to measure the radioactivity remaining in the discs. For discs containing \sim 100 μ g protein, the efficiency of counting was \sim 20%. Approximately 80% of the radioactivity loaded in gradients was recovered in the fractions.

(j) *Amino Acid Incorporation In Vitro*

10- to 12-day old chicken embryos were used to prepare pH 5 and Sephadex G-100 (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) fractions (Blobel and Potter, 1967). The pH 5 fraction was diluted with solution A to have \sim 8 OD280 units/ml, and the G-100 fraction to have \sim 6 OD280 units/ml.

The incorporation medium contained in 1 ml: 400 μ l of ribosome solution (75-225 μ g/ml) from 9- to 12-day old embryos or solution A (as a control), 50 μ l of 0.02 M adenosine triphosphate (ATP), 50 μ l of 0.01 M guanosine triphosphate (GTP), 50 μ l of 0.20 M phosphoenol pyruvate, 5 μ l of pyruvate kinase (10 mg/ml, [Calbiochem, Los Angeles, Calif.], 50 μ l of U-phenylalanine-1-¹⁴C solution (10 μ Ci/ml, SA 375 mCi/mmol, [New England Nuclear]), 100 μ l of pH 5 fraction, 190 μ l of G-100 fraction, 10 μ l of polyuridylic acid (poly U) (1 mg/ml, [Miles Laboratories Inc., Elkhart, Ind.]) or distilled water, and 100 μ l of a compensating buffer to adjust the final ionic concentration to 100 mM KCl, 50 mM Tris-HCl (pH 7.5), and various concentrations of Mg⁺⁺. The components were mixed at 0°C, and the reaction was started by transferring to a water bath at 37°C. At desired times, 100 μ l portions were pipetted onto Whatman 3 MM filter paper discs which were processed as described by Mans and Novelli (1960). The hot acid-insoluble radioactivity in the discs was measured by liquid scintillation counting in 10 ml of Liquifluor.

(k) *Protein, RNA, and Radioactivity Determinations*

Protein and RNA were extracted according to the procedure of Siekevitz (Siekevitz, 1952). The protein residue was dissolved in 0.5 ml of 0.1 N NaOH and diluted with water to 1 ml. The radioactivity in 0.5 ml portions was measured by liquid scintillation

counting in 10 ml of Bray's solution (Bray, 1960). The remaining alkaline digest was used for protein determinations according to Lowry et al. (1951), using crystalline bovine serum albumin as a standard. RNA was measured by the orcinol method (Mejbaum, 1929), using yeast RNA (Sigma Chemical Co., St. Louis, Mo.) as a standard.

(l) Protein Electrophoresis

Purified ribosomal subunits were prepared from tetramers and from polysomes dissociated in 500 mM KCl, 50 mM TEA, 5 mM MgCl₂ containing 5 × 10⁻⁴ M puromycin. Proteins were extracted, reduced, and alkylated by the procedure of Maizel (1969) for SDS polyacrylamide gel electrophoresis. Neutralized samples containing ~150–200 mg protein were layered onto 15% discontinuous polyacrylamide gels containing sodium dodecyl sulfate. Electrophoresis was carried out at 45 v and 1 ma/gel for 16 hr at room temperature. Gels were stained with 1% buffalo black in a methanol:acetone:water mixture (50:10:40) for 12–24 hr at room temperature and destained with 70% acetic acid at 37°C. The absorbance in the visible range was traced along the gels with a Densicord recording electrodensitometer model 542 (Photovolt Corporation, New York).

RESULTS

(1) State of Aggregation of Ribosomes after Slow Cooling

Approximately 65% of the RNA in the post-mitochondrial supernatant from either control or cooled 11-day old embryos was recovered in samples of crude ribosomes (Table I). In control samples most ribosomes exist as polysomes, in which up to 14 different components could be resolved (Fig. 1 a). Hypothermic treatment for 24 hr produced two main changes in the sedimentation profile of crude ribosomes (Fig. 1 b): (1) a decrease in the amount and average size of the polysomes, and (2) the appearance of a prominent peak (representing ~20% of the area of total ribosomes) corresponding to a component with a sedimentation coefficient of ~166S (calculated by using the position of the 80S monomer as a reference [Martin and Ames, 1961]). Components with similar sedimentation coefficients have previously been described in homogenates obtained from embryonic back skin and feathers incubated in cold media (Bell et al., 1965) and in ribosomes

TABLE I
Protein and RNA Content of Fractions from Cooled Embryos*

Fractions	Protein	RNA	Total Ribosomes released by puromycin-KCl‡
	%	%	%
Total homogenate§	100 (37,000 µg)	100 (1900 µg)	100 (2730 µg)
Nuclear fraction§ (washed)	27	33	15
Mitochondrial fraction (washed)	14	13	10
Postmitochondrial supernatant	57	51	72
Crude ribosomal fraction from postmitochondrial supernatant	2	34	49

* 11-day old embryos from eggs cooled for 24 hr were homogenized and fractionated as described in Materials and Methods. The wet weight of starting materials was 21 g.

‡ Fractions were incubated at 37°C for 10 min in 500 mM KCl–50 mM TEA–5 mM Mg⁺⁺ containing 5 × 10⁻⁴ M puromycin to release and dissociate all ribosomes into subunits (Blobel and Sabatini, 1971; Adelman et al., 1970). Homogenate and nuclear fractions also received pancreatic DNase (25 µg/ml). After incubation at 37°C for 10 min, all samples were brought to 25 mM MgCl₂, cooled to 0°C, and further incubated for 15 min at 0°C to complete digestion of DNA which in homogenate or nuclear fractions might be released by the higher salt treatment. The samples were layered onto 12 ml, 5–20% sucrose density gradients containing 500 mM KCl–50 mM TEA–5 mM MgCl₂. The gradients were centrifuged at 40,000 rpm and 4°C for 3½ hr. The amount of ribosomes was calculated from the optical density at 260 mµ of fractions corresponding to ribosomal subunits.

§ The homogenate and the nuclear fraction contain unbroken cells, which do not release their ribosomes by the puromycin-KCl procedure.

|| Microgram per gram wet weight of embryo.

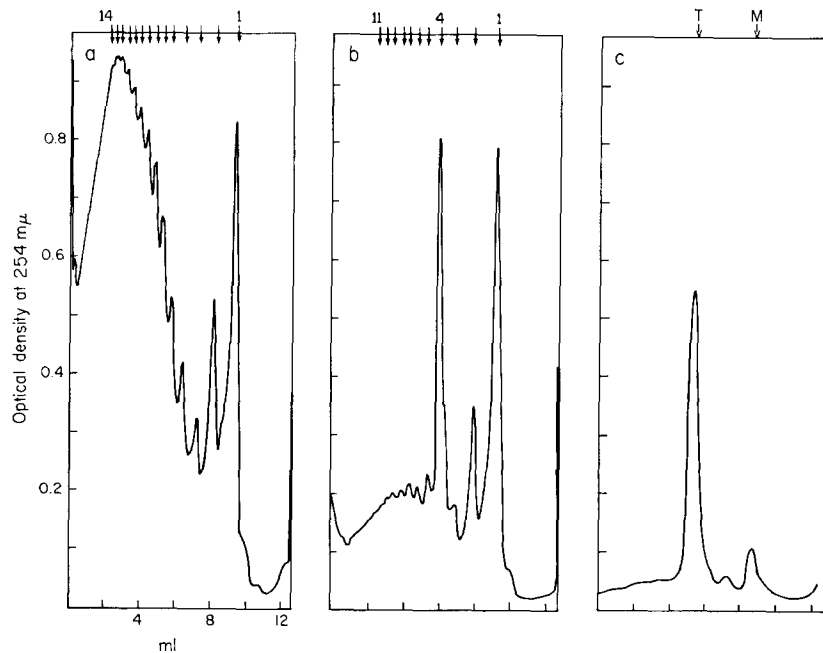


FIGURE 1 Sedimentation profiles of (a) normal ribosomes; (b) ribosomes from cooled eggs; and (c) isolated tetramers from 11-day old chicken embryos. 10–40% sucrose density gradients were made with the ionic composition of solution A. (a) and (b) received 7 and 4 OD260 units of ribosomes, respectively, and were centrifuged at 40,000 rpm for 100 min. (c) received 0.9 OD260 units of purified tetramers and was centrifuged at 40,000 rpm for 150 min. *T* and *M* indicate tetramer and monomer positions.

obtained from cooled 7-day old fertilized eggs (Carey, 1970). The sedimentation pattern of a purified sample of ~166S components prepared as described in Materials and Methods (section *e*) is shown in Fig. 1 *c*. The peak at 166S is symmetric and includes ~90% of the material in the sample. Electron microscope examination² showed that the 166S particles in these samples are square tetramers of rounded particles ~280 Å in diameter with a characteristic 80S ribosome fine structure. This type of preparation, referred to as tetramer sample, was used to characterize biochemically the ~166S particles and to assay *in vitro* their amino acid incorporation activity.

(2) Tetramer Formation at Different Stages of Development

In Fig. 2 the relative amount of tetramers present in the postnuclear supernatant of embryos cooled for 24 hr is correlated with the state of

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

growth, represented by the wet weight and RNA content of the embryos. The proportion of ribosomes involved in tetramer formation decreases rapidly with age from ~35% at 3 days to ~18% at 13 days. For this reason, young embryos 5 days old were used for the studies of the kinetics of crystallization *in vivo* reported in the following paper. For preparative purposes, however, 10- to 12-day old embryos were selected in order to reduce the number of eggs needed.

(3) Characterization of Ribosome Tetramers

(a) DISSOCIATION OF TETRAMERS INTO RIBOSOMAL SUBUNITS: To prove that the 166S particles consist of 80S ribosomes which can be dissociated into normal 60S and 40S subunits, purified samples were incubated for 10 min at 37°C in a solution containing 0.5 M KCl–0.05 M Tris-HCl (pH 7.5)–0.005 M Mg⁺⁺ (HSB) with or without 5×10^{-4} M puromycin. Incubation in HSB caused the complete dissociation of tetramers (Fig. 3 *b*) into 60S and 40S particles with a weight

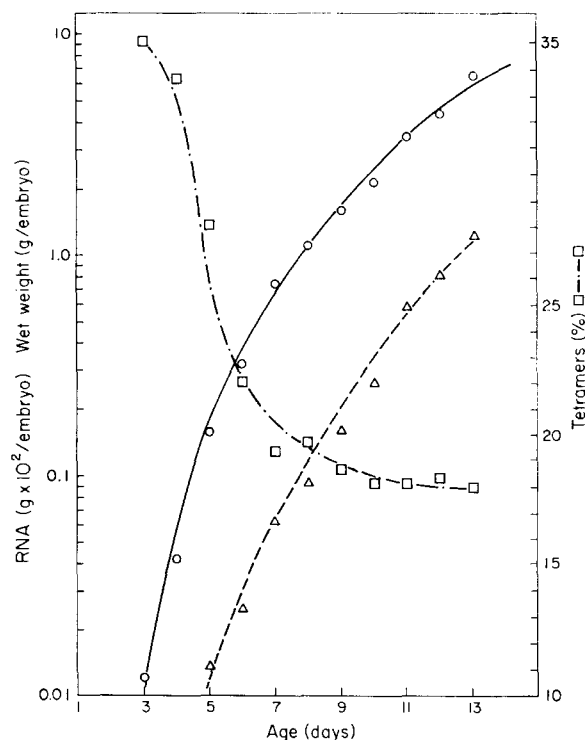


FIGURE 2 Tetramer formation at different stages of development. After cooling for 24 hr, embryos of different ages were removed, washed in solution B, weighed, and homogenized. Portions of the homogenate were used for RNA determinations and the rest for the preparation of a postnuclear supernatant (600 g for 5 min). After treatment with 10 μ g RNase at 0°C for 10 min to convert polysomes into monomers, portions of the postnuclear supernatant were loaded onto sucrose density gradients, as in Fig. 1, and centrifuged for 120 min at 40,000 rpm. The proportion of tetramers is shown as percentage of the total ribosomes displayed in the sedimentation pattern. Wet weight (-O-O-O-) and RNA content (- Δ - Δ -) per embryo are expressed in a logarithmic scale.

ratio of $\sim 2.4:1$, as estimated from the optical density profiles, whether or not puromycin was added. On the other hand, puromycin is needed to produce the dissociation of normal polysomes into ribosomal subunits (Fig. 3 a) (Blobel and Sabatini, 1971).

(b) RNA IN TETRAMERS: An examination by sedimentation analysis (Figs. 3 c, d) and polyacrylamide gel electrophoresis (not shown) of the classes of RNA within RNA extracts from tetramers and polysomes shows that both types of ribosomes contain similar species of rRNA's (28S, 18S, and 5S) in their normal proportions. The amount of 4S RNA in tetramer samples, however, estimated in composite gels stained with methylene blue was much smaller than that in polysomes. This was confirmed by analyzing in 5% acrylamide gels the material released from tetramers treated with EDTA (Fig. 4). Tetramers released as

much 5S RNA as, but much smaller amounts of 4S RNA ($\sim 20\%$), than did an equivalent amount of polysomes. Since both 4S and 5S RNA's are released from ribosomes treated with EDTA (Petermann and Pavlovic, 1969), and since each large subunit contains one 5S RNA molecule, the 4S RNA detected in tetramer samples amounts to much less than one molecule per ribosome and probably represents polysomal contamination, and/or tRNA adsorbed during preparation procedures.

(c) PROTEINS IN TETRAMERS: Results of the electrophoretic analysis (Fig. 5) demonstrate that the proteins of large and small subunits derived from polysomes or from tetramers are similar in kind and proportion. No major extra protein bands were found in subunits from tetramers, nor were the tetramers deficient in protein content.

(d) INABILITY OF TETRAMERS TO COUPLE

LABELLED PUROMYCIN TO NASCENT POLYPEPTIDES: Although the previous results indicate that tRNA is low, possibly absent from tetramers, it is possible that tetramers contain peptidyl-

tRNA molecules which can be detected in vitro through the coupling of radioactive puromycin.

The in vitro incorporation of radioactive puromycin into hot acid-insoluble peptides by chicken

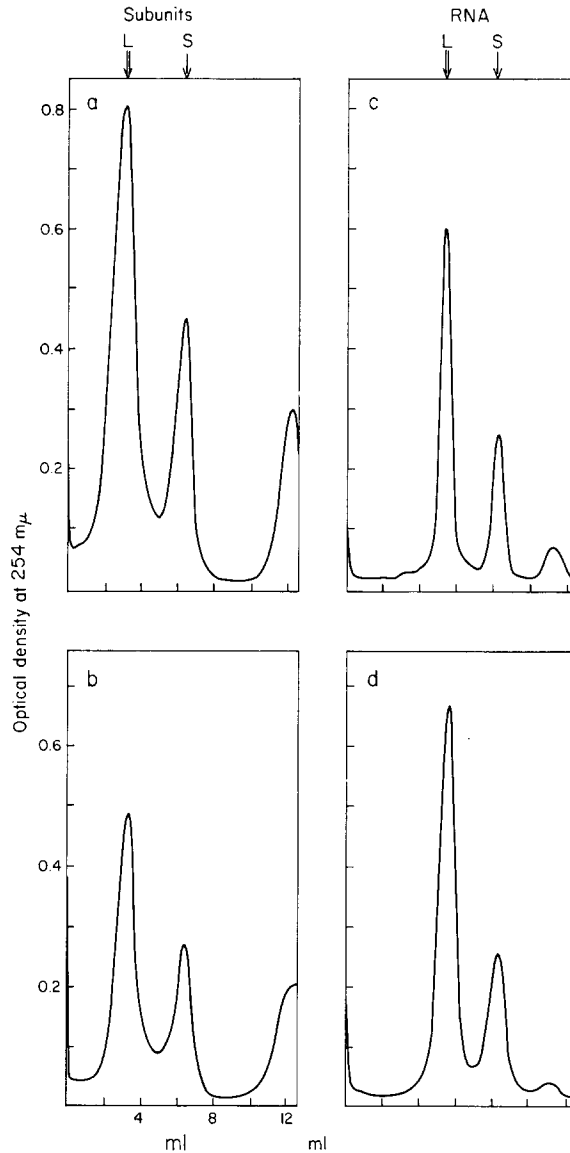


FIGURE 3 Ribosomal subunits and RNA's in polysomes and tetramers. Normal ribosomes (a) and purified tetramers (b) were dissociated into subunits by incubation for 10 min at 37°C in 0.5 M KCl, 0.05 M Tris-HCl (pH 7.5), and 0.005 M Mg⁺⁺ containing 5×10^{-4} M puromycin. 0.3 ml portions containing 14 OD₂₆₀ units of polysomes (a) and 8 OD₂₆₀ units of tetramers (b) were layered on 12.5 ml, 5–20% sucrose density gradients of the same ionic composition of the incubation medium and centrifuged at 20°C for 150 min at 40,000 rpm. RNA from normal polysomes (c) and from tetramers (d) was extracted as described in Materials and Methods, section f. Portions containing ~3 OD₂₆₀ units were layered onto 12.5 ml of 5–20% sucrose density gradients containing 0.1 M NaCl, 0.050 M acetate buffer pH 5.2, prepared in tubes of the SB283 rotor. The gradients were centrifuged at 2°C for 14 hr at 30,000 rpm.

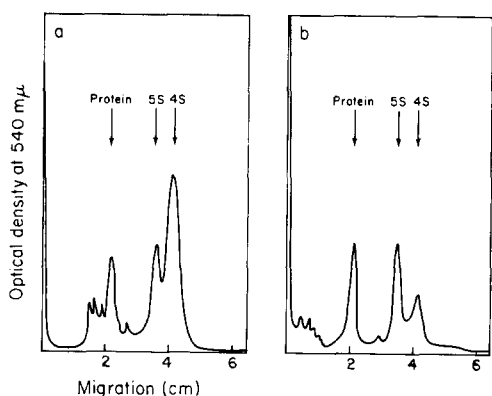


FIGURE 4 Densitometric tracing of acrylamide gel electrophoresis of material released from control polysomes (a) and from tetramers (b) treated with EDTA (Materials and Methods, section f).

embryo polysomes from control eggs is shown in Fig. 6. The reaction shows similar characteristics as in rat liver polysomes (Blobel and Sabatini, 1971). It proceeds at 0°C, does not require the addition of protein factors from the cell sap or an energy supply, and at a constant Mg^{++} concentration is stimulated by an increase in ionic strength. Furthermore, the extent of puromycin coupling is proportional to the amount of polysomes containing the nascent polypeptides. Assuming a molecular weight of 5×10^6 daltons for an 80S ribosome (Tashiro and Yphantis, 1965), it can be calculated that in 1 M KCl chicken embryo polysomes couple (into acid-insoluble peptidyl-puromycin) ~ 0.8 moles of puromycin per mole of ribosomes. Tetramer samples, however, were 10 times less efficient than an equivalent amount

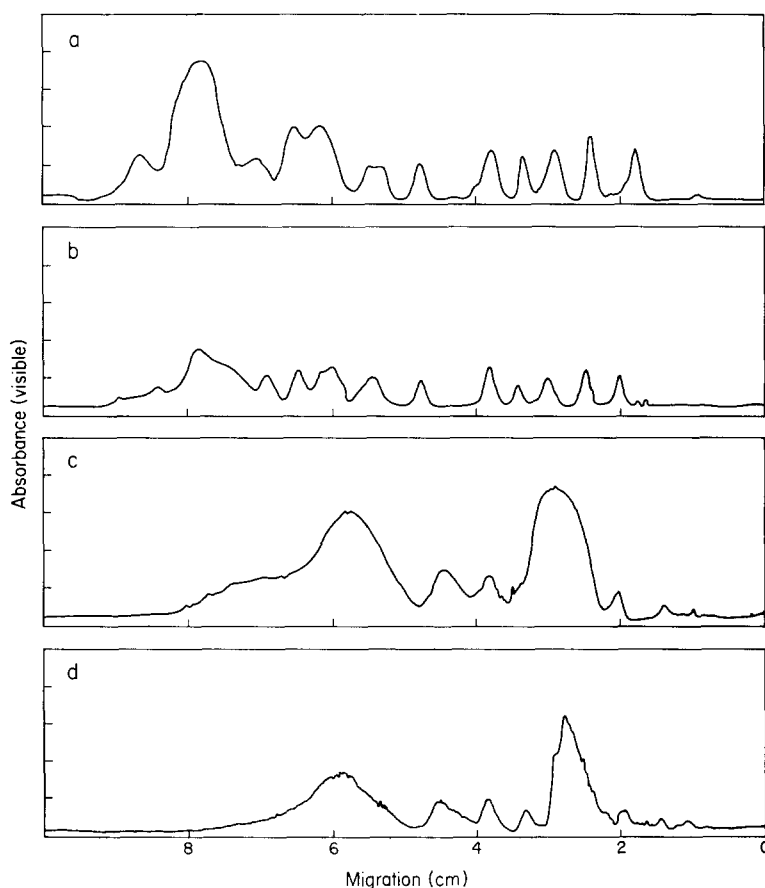


FIGURE 5 Densitometric tracing of proteins analyzed by SDS acrylamide gel electrophoresis. a, b: large subunit proteins from polysomes (a) and tetramers (b). c, d: small subunits proteins from polysomes (c) and tetramers (d). For details see Materials and Methods (section l).

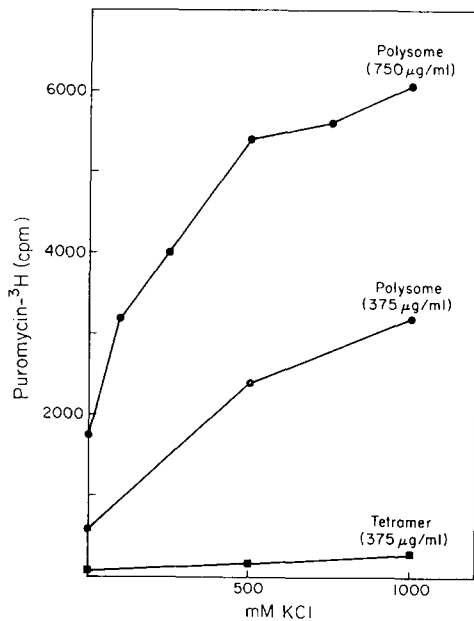


FIGURE 6 In vitro coupling of puromycin- ^3H to nascent polypeptides in polysomes and tetramers. The incubation mixture was described in Materials and Methods (section *g*). After 2 hr at 0°C the radioactivity incorporated into hot acid-insoluble products was measured in $100\ \mu\text{l}$ portions by the filter paper method (Materials and Methods, section *i*).

of polysomes (Fig. 6), and their low coupling activity can be attributed to contaminating polysomes since the purity of the tetramer samples was shown (Fig. 1 *c*) to be $\sim 90\%$. It follows that nascent polypeptides capable of reacting with puromycin are absent from tetramers.

(*e*) SENSITIVITY OF TETRAMERS TO RNASE TREATMENT AT 0°C AND AT DIFFERENT Mg^{++} CONCENTRATIONS. It has been reported (Humphreys et al., 1964; Bell et al., 1965) that tetramers derived from back skin and feathers of chicken embryos are resistant to RNase treatment in a buffer containing 10 mM KCl–10 mM Tris (pH 7.5)–1.5 mM Mg^{++} . We have examined the effect of pancreatic RNase (Worthington Biochemical Corp., Freehold, N. J.) on tetramers at 0°C and at different Mg^{++} concentrations, since Mg^{++} is an important factor in preserving the conformation of ribosomes. Ribosomes from control and cooled embryos were used for these experiments so that the stability of the tetramers could be compared to that of polysomes from control as well as cooled samples. At 5 mM Mg^{++}

(Figs. 7 *a, b*), polysomes incubated with RNase for 10 min at 0°C were almost completely degraded to monomers and dimers, while tetramers were undissociated (Figs. 7 *c, d*).

It should be noted that a reduction in the Mg^{++} concentration alone (Figs. 7 *c, 8 a, b, c*) from 5 mM to 1 mM did not greatly change the amount of polysomes and dimers so that, at 1 mM Mg^{++} , mostly tetramers and monomers were present. The effect of RNase on tetramers clearly depends on the Mg^{++} concentration (Figs. 7 *d, 8 d, e, f*): the enzyme has no detectable effect at 5 mM Mg^{++} (Figs. 7 *c, d*), but causes progressive decrease of tetramers at lower concentrations until at 1 mM Mg^{++} almost all the ribosomes from cooled eggs are converted into monomers (Fig. 8 *f*).

The insensitivity of tetramers to RNase treatments at 0°C which convert polysomes to monomers suggests that mRNA is not involved in joining the ribosomes within this aggregate. The effect of RNase at Mg^{++} concentrations lower than 2 mM is probably due to a change in ribosome conformation within the tetramers which makes rRNA accessible to the enzyme. It should be noted that in the previous work of Humphreys et al. (1964), resistance to RNase at 1.5 mM Mg was observed, but the ionic strength used was considerably lower than in our experiments.

(*f*) EFFECT OF Mg^{++} AND KCl CONCENTRATIONS ON THE STABILITY OF TETRAMERS: The ratio of monovalent to divalent cations is important in maintaining the conformation of ribosomal subunits and their association within monomers (Petermann, 1964). Since it may also affect interribosome binding, tetramers were incubated in a series of solutions in which the concentrations of MgCl_2 and KCl were varied independently. After incubation, the samples were fixed in formaldehyde to prevent further changes in particle association during zone centrifugation.

Fig. 9 shows that the proportion of tetramers in samples incubated for 10 min at 37°C in 50 mM KCl and 50 mM TEA progressively decreased (and the proportion of monomers increased) as the Mg^{++} concentration was reduced below 5 mM, so that at 1 mM Mg^{++} tetramers were completely converted into monomers. Under all conditions represented in Fig. 9, few ribosomal subunits were produced in either tetramer samples or monomer controls.

The effect on tetramers and monomers of raising

the ionic strength while maintaining the Mg^{++} concentration at 5 mM is shown in Figs. 10 *a, b*. It should be noted that up to 100 mM KCl neither the tetramer sample (Fig. 10 *a*) nor the monomer control (Fig. 10 *b*) showed dissociation into sub-

units, although at 100 mM KCl ~ 30% of the ribosomes in the tetramer sample were monomers. At higher concentrations of KCl, both samples contained subunits in a proportion which was related to the amount of monomers present. For

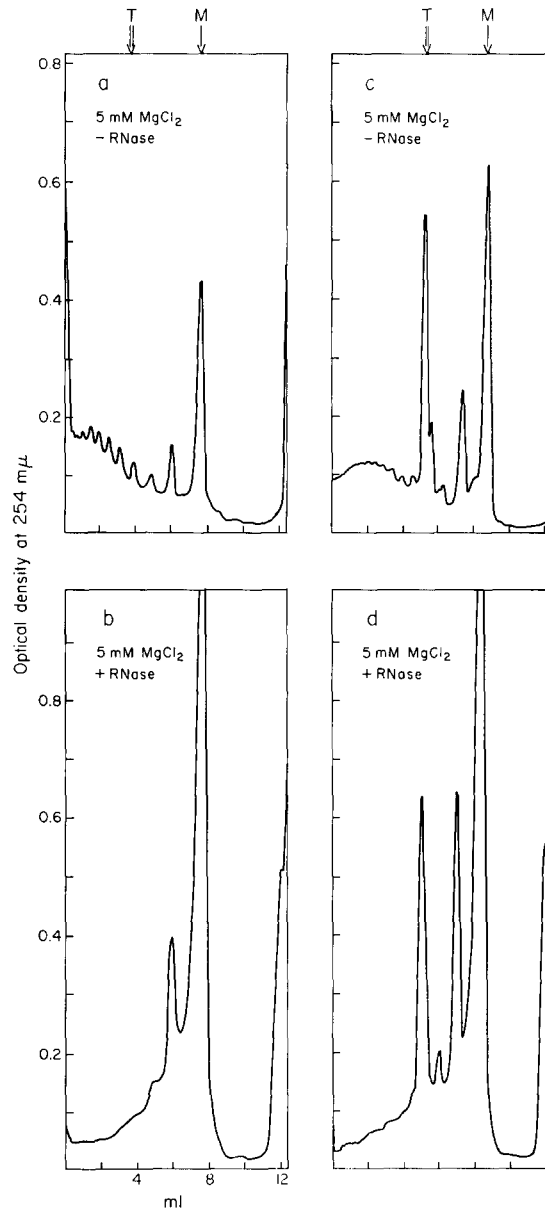


FIGURE 7 Effect of RNase on polysomes and tetramers at 5 mM Mg^{++} . Normal polysomes (*a, b*) and ribosomes from cooled eggs (*c, d*) were resuspended in solution A. 0.3 ml portions containing 4 OD₂₆₀ units were incubated for 10 min at 0°C with (*b, d*) or without (*a, c*) 5 μ g RNase. The samples were layered onto 12.5 ml, 10–40% sucrose density gradients in solution A, which were centrifuged for 120 min at 40,000 rpm.

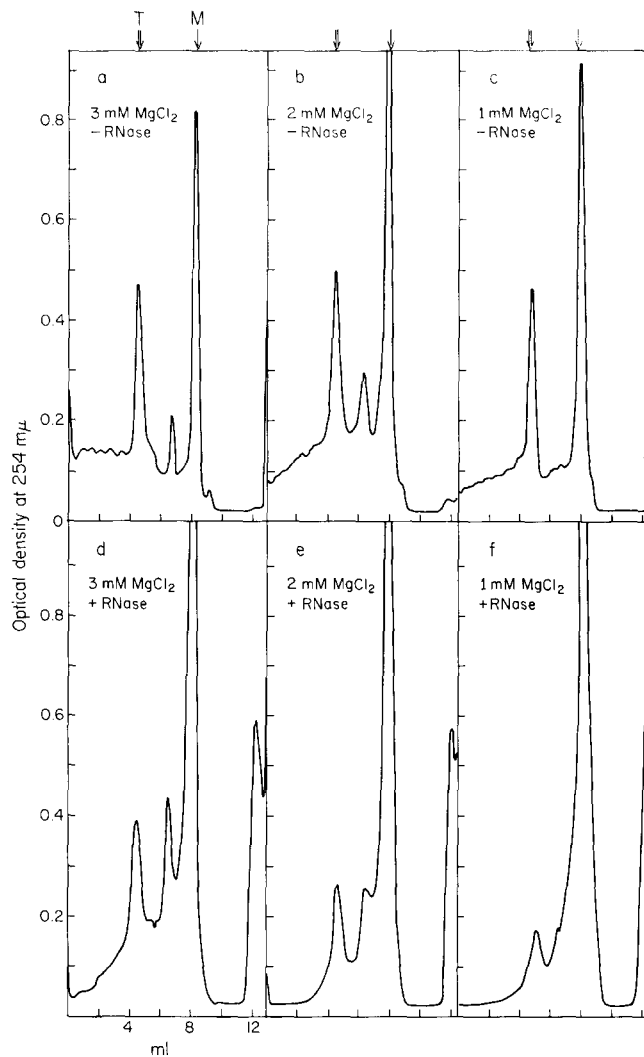


FIGURE 8 Stability of tetramers to RNase treatment at various concentrations of $MgCl_2$. Ribosomes from cooled eggs were resuspended in 0.050 M KCl - 0.050 M Tris-HCl (pH 7.5) containing 0.003 M (a, d), 0.002 M (b, e), or 0.001 M (c, f) $MgCl_2$. The samples were incubated with (d, e, f) or without RNase (a, b, c) for 10 min at 0°C , loaded on sucrose density gradients of the same ionic composition and analyzed as described in the legend to Fig. 7.

example, at 175 mM KCl - 50 mM TEA-HCl (pH 7.5), most tetramers (87%) were disassembled, but only half of the resulting monomers were dissociated into subunits (Fig. 10 a). Under these conditions, $\sim 50\%$ of the control monomers are equally converted into subunits (Fig. 10 b). The results in Figs. 9 and 10 a, b show that the inter-ribosome binding within tetramers is more sensitive to lowering the Mg^{++} concentration or raising the ionic strength than the binding in between subunits and suggest that tetramers were disassembled into monomers without previous dis-

sociation into subunits. They also show that monomers derived from tetramers dissociate into subunits at approximately the same ionic conditions as control monomers.

(4) *In Vitro* Amino Acid Incorporation Activity of Tetramers

The activity of tetramers and control ribosomes in protein synthesis was tested *in vitro*, using either endogenous or artificial (poly U) programming.

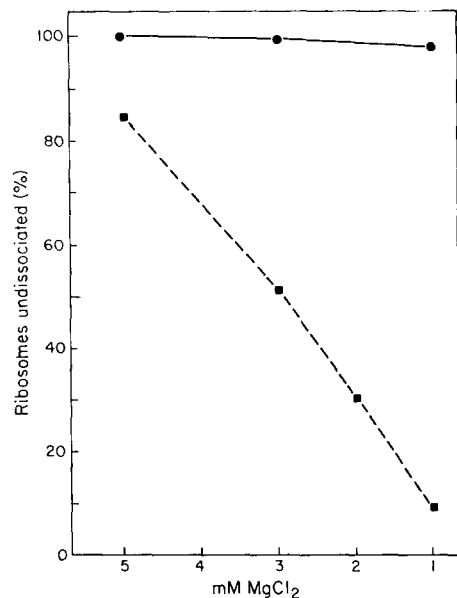


FIGURE 9 Stability of tetramers and normal monomers at different Mg^{++} concentrations. Purified tetramers and control monomeric ribosomes were dialyzed for 3 hr at $0^{\circ}C$ against 0.050 M KCl - 0.050 M TEA ($pH\ 7.5$) with the Mg^{++} concentrations indicated. Portions (0.6 ml , $\sim 1.2\text{ OD}_{260}$ units) were incubated at $37^{\circ}C$ for 10 min and fixed at room temperature for 5 min in 3% formaldehyde. The samples were analyzed in 12 ml , 10 - 40% sucrose density gradients of the same ionic composition as the solution of dialysis. The proportion of tetramers (■-■-■) and monomers (●-●-●) was calculated after centrifugation at $2^{\circ}C$ for 140 min at $40,000\text{ rpm}$ and is expressed as percentage of the amount of tetramers or monomers present in 0.050 M KCl - 0.050 M TEA ($pH\ 7.5$)- 0.005 M Mg^{++} before incubation at $37^{\circ}C$.

The results in Fig. 11 *a* show that: (1) normal polysomes have considerable endogenous activity which is optimized at Mg^{++} concentrations that range from 5 to 15 mM . (2) These polysomes can also be programmed by poly U—with an optimum at a Mg^{++} concentration within the range of 10 - 25 mM —without the need of a previous incubation to eliminate their endogenous activity. (3) Tetramers, as opposed to polysomes, show no endogenous activity at all Mg^{++} concentrations from 5 to 35 mM , but could be programmed by poly U with an optimum at a Mg^{++} concentration of 25 mM (Fig. 11 *a*). These facts can be analyzed in more detail in Figs. 11 *b*, *c* which show the kinetics of incorporation of phenylalanine- ^{14}C by tetramers and by polysomes incubated with and without poly U at 5 mM (Fig. 11 *b*)

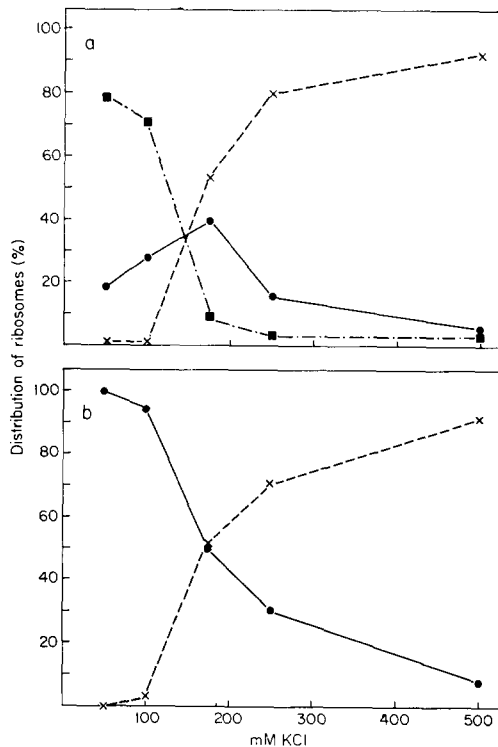


FIGURE 10 Stability of tetramers and normal monomers in solutions of increasing ionic strength. Purified tetramers (*a*) and control monomeric ribosomes (*b*) were dialyzed against 0.050 M KCl - 0.050 M TEA ($pH\ 7.5$)- 0.005 M Mg^{++} as described in Fig. 9. 0.6 ml portions containing 1.2 OD_{260} units of ribosomes received a compensating solution to adjust the KCl concentration to the values indicated. The samples were incubated for 10 min at $37^{\circ}C$, fixed, and analyzed as described in Fig. 9. The percentages of tetramers, monomers, and subunits after each incubation are expressed with reference to a nonincubated sample kept at $0^{\circ}C$ in 0.050 M KCl - 0.050 M TEA ($pH\ 7.5$)- 0.005 M Mg^{++} . ●-●-●, percentage of monomers; ■-■-■, percentage of tetramers; X---X---X, percentage of subunits.

and at 25 mM (Fig. 11 *c*) Mg^{++} . It is clear that at 5 mM Mg^{++} (Fig. 11 *b*) poly U has no stimulating activity on polysomes, while at 25 mM Mg^{++} the incorporation of phenylalanine by polysomes is more than 20 times higher than the endogenous activity, which is considerably suppressed at this Mg^{++} concentration. Tetramers have no endogenous activity at 5 mM or at 25 mM Mg^{++} , and can not be programmed by poly U at 5 mM Mg^{++} , but accept poly U at 25 mM Mg^{++} , becoming as active in the incorporation of phenylalanine- ^{14}C as normal polysomes (incubated with poly U).

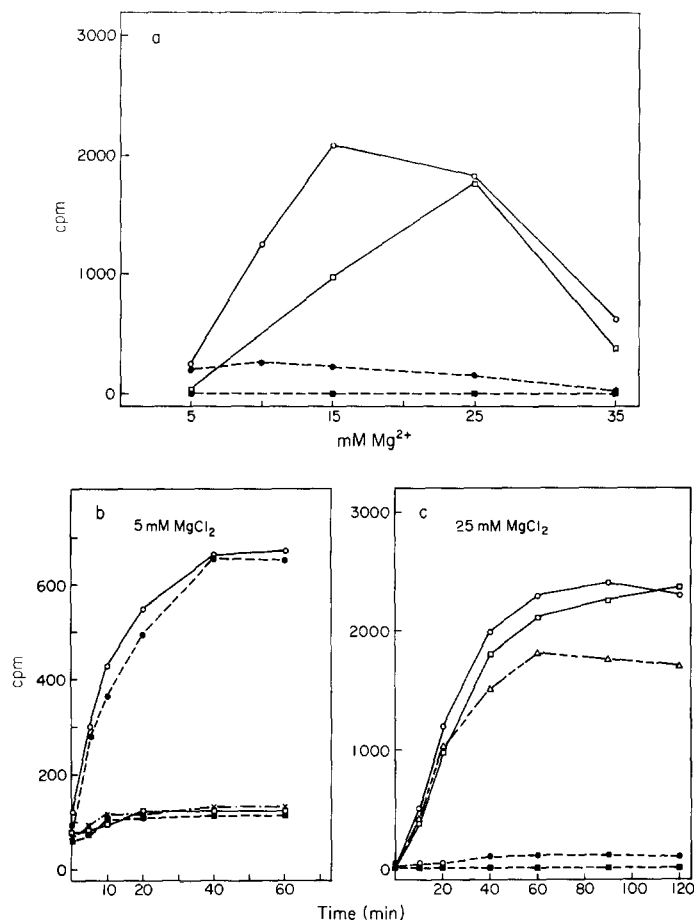


FIGURE 11 Endogenous and poly U-directed phenylalanine- ^{14}C incorporation by tetramers and polysomes. (a) Effects of various concentrations of $MgCl_2$. Each point corresponds to the radioactivity in $\sim 7.5 \mu g$ of ribosomes in 0.1 ml of incubation mixture measured after 40 min of incubation (see Materials and Methods, section j). (b) Lack of stimulation by poly U at 5 mM $MgCl_2$. Each point corresponds to the radioactivity in $22.5 \mu g$ of ribosomes in 0.1 ml of incubation mixture. (c) Poly U-directed phenylalanine- ^{14}C incorporation at 25 mM $MgCl_2$. Each point corresponds to the radioactivity in $7.5 \mu g$ of ribosomes in 0.1 ml of incubation mixture. $\circ-\circ-\circ$ and $\bullet-\bullet-\bullet$, polysomes, with and without poly U; $\square-\square-\square$ and $\blacksquare-\blacksquare-\blacksquare$, tetramers with and without poly U; $\triangle-\triangle-\triangle$, monomers derived from tetramers (see text) with poly U; $\times-\times-\times$, no ribosomes with poly U.

It should also be noted that tetramers at 25 mM Mg^{++} are more active than monomers derived from tetramers by previous incubation at 37°C for 10 min in 50 mM KCl-1 mM Mg^{++} -50 mM Tris-HCl (pH 7.5). The kinetics of incorporation also show that within the conditions of the experiments there is no lag in the course of incorporation of phenylalanine by tetramers, suggesting that tetramers could be programmed by poly U without previous dissociation into monomers or subunits.

It should be noted that these experiments were performed with tetramer samples, which as shown in Fig. 1 were, before the beginning of incubation

at 37°C, more than 90% pure. Therefore, it was important to establish whether the ribosomes in the tetramer samples were being programmed by poly U as tetramers or as monomers or subunits after dissociation during incubation.

(5) Phenylalanine- ^{14}C Incorporation by Intact Tetramers

Complete mixtures for phenylalanine- ^{14}C in vitro incorporation by tetramers were analyzed in sucrose gradients at the end of incubation. The results (Fig. 12) showed that after incubation for

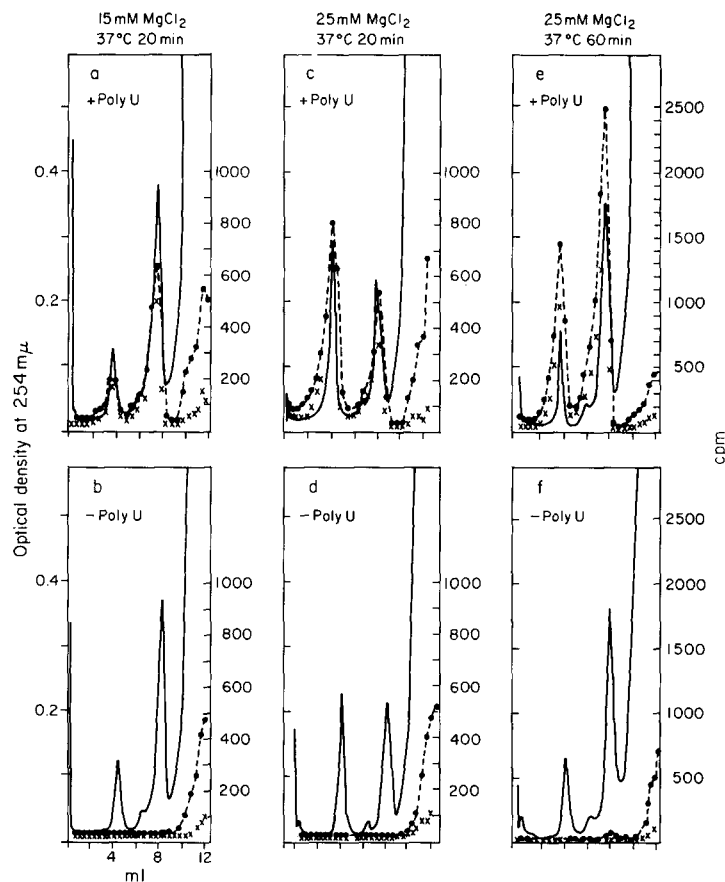


FIGURE 12 Phenylalanine- ^{14}C incorporation by tetramers programmed with poly U. 0.8 ml portions from incubation mixtures containing $\sim 60 \mu\text{g}$ of tetrameric ribosomes (see Materials and Methods, section *j*) were layered onto 12 ml, 10–40% sucrose density gradients of the same ionic composition as the incorporation medium and centrifuged at 40,000 rpm and 2°C for 150 min. Hot ($\bullet\text{---}\bullet\text{---}\bullet$) and cold ($\times \times \times \times$) acid-insoluble radioactivities throughout the gradients were measured to estimate the content of nascent polypeptides and of amino acyls tRNA, respectively, as described in Materials and Methods (section *i*).

20 min at 37°C with 25 mM Mg^{++} (Fig. 12 *c*), 50% of the tetramers in the incorporation mixture were converted into monomers. The proportion of initial tetramers which dissociated into monomers depended on the time of incubation at 37°C ($\sim 70\%$ dissociation after 1 hr in a medium containing 25 mM Mg^{++} , Fig. 12 *e*) and on the concentration of Mg^{++} ion ($\sim 70\%$ dissociation after only 20 min at 37°C in a medium containing 15 mM Mg^{++} , Fig. 12 *a*). Control experiments showed that incubation for up to 1 hr at 0°C produced almost no tetramer dissociation. Figs. 12 *b*, *d*, *f* demonstrate that the conversion of tetramers into monomers which proceeds in the incorporation mixture during incubation at 37°C does not

depend on protein synthesis, since it occurred to the same extent when poly U was omitted and phenylalanine incorporation was null.³ The distribution throughout the gradients of the acid-insoluble phenylalanine- ^{14}C radioactivity incorporated during incubation with poly U clearly shows (Figs. 12 *a*, *c*, *e*) that both, monomers and tetramers, contained labeled polyphenylalanine. Moreover, the specific activity of tetramers (cpm

³ We established that some components in the incorporation medium by themselves accelerated the dissociation of tetramers into monomers at 37°C (for example, phosphoenol pyruvate but not ATP or GTP).

of phenylalanine- ^{14}C per OD260 $m\mu$) after 20 min of incubation at 37°C was higher at 25 mM Mg^{++} (1880 cpm/OD260) than at 15 mM Mg^{++} (1610 cpm/OD260), and, at all times of incubation, was 25–35% lower in monomers than in tetramers.

These results indicate that tetramers are capable of incorporating polyphenylalanine without previous dissociation into monomers or subunits, and that monomers released from tetramers are also capable of phenylalanine incorporation.

DISCUSSION

Ribosome samples from different tissues of hypothermically treated chicken embryos contain a population of ribonucleoprotein particles which have a sedimentation coefficient of $\sim 166\text{S}$ (Humphreys et al., 1965; Carey, 1970). Electron microscope observations² indicate that these particles—which in 5-day old embryos cooled for 24 hr constitute $\sim 30\%$ of the ribonucleoprotein—are tetrameric units derived during homogenization from cytoplasmic, two-dimensional crystalline ribosome lattices, first described by Byers (1966, 1967). Several observations led us to conclude that these tetramers are formed by mature 80S ribosomes. Purified samples of tetramers incubated at 37°C in solutions of high ionic strength disassemble into equimolar amounts of 60S and 40S subparticles, similar to normal ribosomal subunits. Acrylamide gel electrophoresis and sedimentation analysis of the RNA extracted from the tetramers showed that these particles contain 28S, 18S, and 5S rRNAs, but little or no 4S RNA. Polyacrylamide gel electrophoresis analysis indicates that a normal complement of ribosomal proteins is present in tetramers.

To elucidate the nature of the interribosome binding within tetramers, we first investigated the possibility that it is mediated through messenger RNA. Several observations led us to conclude that this is not the case. At Mg^{++} concentrations higher than 2 mM tetramers are resistant to RNase treatment at 0°C , while polysomes are sensitive to RNase at all Mg^{++} concentrations. Furthermore, experiments on the incorporation of orotic- ^3H acid into cooled eggs (reported in the following paper) failed to detect any species of newly synthesized RNA within the tetramers. Another distinction between tetramers and polysomes is that, by changing the ionic conditions, the interribosome binding in tetramers can be broken independently of the binding between subunits

within monomeric ribosomes. This observation suggests that the interribosome binding within tetramers, although of ionic nature, is probably weaker than the binding between subunits of the tetrameric ribosomes.

The more direct demonstration that tetramers are not functional units and contain ribosomes which are not programmed for protein synthesis is their complete lack of endogenous activity in an *in vitro* system for the incorporation of amino acids. Not only were tetramers inactive under conditions which led to high levels of incorporation by polysomes, but they lacked activity at all Mg^{++} concentrations tested from 5 to 35 mM. Additional evidence indicating that tetramers lack peptidyl-tRNA is their inability to perform the *in vitro* coupling of puromycin- ^3H into acid-insoluble products, and their behavior *in vitro* in solutions of high ionic strength. Tetramers dissociated into ribosomal subunits during incubation at 37°C in 500 mM KCl, 50 mM TEA-HCl (pH 7.5), 5 mM Mg^{++} . The dissociation of true (active) polysomes in the same medium requires puromycin (Martin and Hartwell, 1970; Blobel and Sabatini, 1971) to uncouple tRNA from the nascent chains.

The high viability of fertilized eggs after 24 hr of cooling (Byers, 1966) and the fact that degradation products of ribosomes were not detected upon rewarming while polysomes reappeared suggest that ribosomes within tetramers can reinitiate protein synthesis and, although inactive, are potentially functional. The capacity of tetramers of incorporating phenylalanine *in vitro* under the direction of poly U supports this contention. One should note, however, that polyphenylalanine synthesis can only be assayed at the high Mg^{++} concentrations needed for stimulation with poly U. Hence, the possibility cannot be ruled out that this Mg^{++} concentration also reverses structural alterations, on account of which tetrameric ribosomes are inactive under standard conditions of incubation.

Of particular interest is the conclusion that tetramers can accept poly U, as well as factors participating in protein synthesis, without previous dissociation into subunits. This conclusion is based on the following findings: (a) The specific activity of incorporation in tetramers was, at all times of incubation, higher than that in the monomers derived from them, and (b) no initial time lag in the course of incorporation was de-

tected as would have been expected from the kinetics of tetramer dissociation, if ribosomes from tetramers could only be recruited for incorporation as monomers or subunits. The ability of 80S monomers from rat liver to accept poly U without previous dissociation into subunits has also recently been reported (Falvey and Staehelin, 1970). Furthermore, tetramers were at least as active as monomers or polysomes in polyphenylalanine synthesis, which suggests that every ribosome within a tetramer is capable of accepting poly U. The results clearly demonstrate that poly U can be decoded by tetrameric ribosomes without the need of dissociation into subunits, and that poly U has access to the presumably normal sites of translation in the monomers within tetramers. These observations should be considered in view of electron microscope observations² showing that the space between ribosomal subunits where poly U may be inserted is accessible from each side of the tetramer. The reservation should be made, however, that poly U-directed activity at best only mimics natural protein synthesis. It may occur only because the normal mechanism of accepting mRNA is distorted by high Mg⁺⁺ concentrations.

One can not decide from our experiments whether within a tetramer all ribosomes read the same poly U molecule or whether each translates a different one. Both possibilities should be considered since it can be estimated that the poly U molecules had an average length of 600 A. The kinetics of labeling with phenylalanine do not indicate that ribosomes succeeded one another in utilizing poly U, but this could be due either to the independent use of different poly U molecules or to the simultaneous binding of several ribosomes to one molecule of poly U. An investigation of this question would require kinetic studies at different poly U concentrations and the use of labeled poly U.

ADDENDUM

After this work was completed, a paper appeared in *Proc. Nat. Acad. Sci. U.S.A.* (1971, 68:440) in which B. Byers also demonstrated that tetramers are active in poly U-directed polyphenylalanine synthesis, and can be dissociated into ribosomal subunits at high ionic strength.

Byers (1971) and recently Carey and Read (1971), *Biochem. J.* 121:511, also reported that an increasing concentration of KCl causes a stepwise dissociation of tetramers, in which small subunits are released first

and a tetramer of large subunits only is formed. Under the conditions of incubation and analysis used by us, we detected a dissociation of tetramers into monomers and, only at higher ionic strengths, of monomers into subunits.

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