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Magnesium Precipitation of Ribonucleoprotein Complexes. Expedient Techniques for the Isolation of Undegraded Polysomes and Messenger Ribonucleic Acid†

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ABSTRACT: A procedure is described for the precipitation of ribosomes from crude homogenates of animal tissues. The procedure was developed using chicken oviduct but has been used successfully with tissues from organisms as diverse as slime mold, insect, and mammal. Tissue is homogenized in the presence of detergent (Triton X-100) and a ribonuclease inhibitor, heparin; then after a 5-min centrifugation at 27,000*g*, the ribosomes in the supernatant are precipitated by addition of $MgCl_2$ to 100 mM, and collected 60 min later by a 10-min centrifugation at 27,000*g*. Ribosomes prepared in this way can be easily resuspended; polysomes are undegraded and biologically active, and they are suitable for the immunoprecipitation of a specific polysome class. In addition, EDTA-derived messenger ribonucleoprotein complexes can be precipitated by this method; however, tRNA is not precipitable. The recovery of translatable ovalbumin mRNA from hen oviduct is approximately 80% and the recovery of ribosomes is virtually 100%. Conditions are described in detail for the extraction of translatable ovalbumin mRNA with sodium dodecyl sulfate–phenol–chloroform, and for the removal of contaminating inhibitors of mRNA translation using high salt washes. The magnesium precipitation method is expedient, economical, and well suited for either large-scale isolation of polysomes and mRNA, or for the fractionation of many tissue samples.

The precipitation of ribosomes by Mg^{2+} was first reported by Takanami (1960). This technique has been used subsequently in several laboratories in conjunction with traditional ultracentrifugation methods during ribosome purification (Attardi and Smith, 1962; Gazzinelli and Dickman, 1962; Petermann and Pavlovec, 1963; Iwabuchi *et al.*, 1970). More recently, it has been used instead of ultracentrifugation for the isolation of ribosomes from crude tissue homogenates (Levy and Carter, 1968; Leytin and Lerman, 1969; Lee and Brawerman, 1971; Bitte and Kabat, 1972; Koka and Nakamoto, 1972; Dessev and Grancharov, 1973; Clemens and Pain, 1974). In this paper, the Mg^{2+} precipitation method is further adapted for the quantitative isolation of undegraded polysomes and translatable mRNA from homogenates of chicken oviduct tissue.

Methods

Buffers. Hepes,[1] a 100 mM stock solution, was prepared, adjusted to pH 7.5 with KOH, and treated with diethyl pyrocarbonate (see below). PB, 25 mM Tris–25 mM NaCl–5 mM $MgCl_2$, adjusted to pH 7.5 at room temperature with HCl: a 10X stock solution was prepared, treated with diethyl pyrocarbonate, and then diluted with distilled water. PBM, 25 mM Tris–25 mM NaCl–100 mM $MgCl_2$ (pH 7.5). 0.2PBM, 0.2PB, 1.0PB: buffers PB or PBM with sucrose added to give the molarity indicated; reagent grade sucrose was used but solutions were always treated with diethyl pyrocarbonate. A, buffer PB containing heparin at 1 mg/ml and 2% Triton X-100; prepared from a 10X stock of buffer PB, dry heparin, and a 10% solution of Triton X-100 in distilled water. B, buffer A (4 volumes) diluted with 1 M $MgCl_2$ (1 volume) to yield 0.2 M $MgCl_2$.

Diethyl Pyrocarbonate Treatment. Two drops (about 50 μl) of diethyl pyrocarbonate (Eastman) were added per 100 ml of solution. The mixture was shaken at room temperature and then placed in a boiling water bath for 15–30 min to decompose the remaining diethyl pyrocarbonate. While it was still hot, the solution was shaken vigorously to allow CO_2 and ethanol to escape; this step was repeated several times. Diethyl pyrocarbonate inactivates RNase and it has been used directly during tissue homogenization. Although intact RNA (Fedorcsák *et al.*, 1969) and polysomes can be prepared (Weeks and Marcus, 1969; Anderson and Key, 1971), this procedure destroys ovalbumin mRNA activity, probably due to the alkylation of RNA (Rhoads *et al.*, 1973; R. D. Palmiter, unpublished).

Magnesium Precipitation of Ribosomes. All procedures were at 0–2°. A 10% homogenate of tissue was prepared essentially as before (Palmiter, 1971). For each gram wet weight of finely minced tissue (either fresh or previously frozen at −20°), 9 ml of buffer A was added and the tissue was homogenized in a Dounce homogenizer (Kontes Glass Co.) with several strokes of a loose pestle followed by 10–20 with a tight pestle. The homogenate was centrifuged for 5 min at 27,000g_{max};

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[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; mRNP, messenger ribonucleoprotein.

the supernatant was decanted into another vessel and an equal volume of buffer B was added. After incubation for at least 1 hr, aliquots (8 ml) were layered over 4-ml pads of sucrose (although 0.2PBM was used in most of these studies, 1.0PB is superior for some applications; see text) in 15-ml Corex tubes and centrifuged for 10 min at 27,000g_{max} with either angle or swinging bucket rotors (Sorvall). The supernatant was removed by aspiration part way into the sucrose pad; the upper portion of the tube was washed with distilled water; the wash removed by aspiration; then the tube was inverted to decant remaining sucrose. After a few minutes, the walls were wiped dry with tissues and the pellet was dissolved in a suitable buffer. For resuspension of polysomes or extraction of RNA, the pellet from 0.4 g of tissue was gently homogenized in 2 ml of 20 mM Hepes. For determination of total A_{260} units, the pellet was dissolved in warm 0.5% SDS.

Extraction of RNA. The procedures described below are given in detail because there are many variations of the phenol extraction procedure in the literature with little explanation for them, although Parish (1972) has provided a valuable discussion. Furthermore, many of the procedures have not been tested with recovery of mRNA activity as a criterion. The procedure described below is suitable for extracting active ovalbumin mRNA from resuspended ribosomes, fractions from sucrose gradients, or dilute (less than 10%) homogenates of tissue in buffer A.

The sample in 2 ml of buffer A, PB, or 20 mM Hepes in a 15-ml Corex tube was diluted 1:1 with 0.1 M NaOAc (pH 5); SDS was added to 0.5% and the mixture vortexed for a few seconds. All subsequent steps were performed at room temperature in order to kkeep the SDS in solution. Liquified phenol (4 ml) was added, a silicone stopper was inserted, the mixture was shaken for a few seconds, and shaken again a few minutes later, then 4 ml of chloroform was added and the mixture shaken again. The mixture was centrifuged briefly to separate the phases (1 min at 15,000g is adequate). The upper aqueous phase should be clear; there should be a flocculent interphase; and the organic phase should be clear or cloudy depending on the temperature of the centrifuge. The lower phase was withdrawn by aspiration through a Pasteur pipet, leaving the interface behind, and discarded; it was replaced with 8 ml of chloroform; the mixture was shaken and centrifuged as before. The chloroform was removed and the chloroform extraction repeated until the interface either disappeared or no longer changed, usually only once more. Then the upper aqueous phase was withdrawn, leaving any interface behind, and the RNA was precipitated with 2 volumes of ethanol at −20°. After sitting overnight at −20°, RNA at a final concentration exceeding 10 μg/ml can be collected quantitatively by a 10-min centrifugation at 27,000g_{max}.

Some of the important aspects of this SDS–phenol–chloroform extraction procedure are discussed below. An acid pH was chosen because it minimizes RNase activity (Brown, 1967), allows EDTA to be used if desired (at alkaline pH EDTA precipitates in ethanol), promotes the rapid removal of the interface material, and minimizes DNA recovery. The salt concentration was chosen so that there would be sufficient salt for ethanol precipitation of RNA; potassium was avoided because it precipitates dodecyl sulfate. No advantage of using divalent cation chelators during the extraction has been observed. Commercial, liquified phenol is suitable without redistillation unless it is colored. It is important to separate the RNA and protein by shaking the sample with phenol before chloroform is added, especially when crude homogenates or concentrated solution of ribosomes are being extracted; otherwise, substantial losses of RNA are incurred, probably due to formation of insoluble protein–RNA aggregates. There are multiple advantages of using chloroform. First, it makes the organic phase sufficiently dense so that it is always at the bottom, even when extracting dense sucrose fractions. Its use also allows a more rapid separation of the phases and permits easy removal of the aqueous phase at the end, thereby minimizing the transfer of phenol, which in turn reduces the need to do repeated ethanol precipitations. Second, phenol alone retains about 10–15% aqueous phase which means that with each extraction a similar loss of RNA occurs. Chloroform prevents this retention of water and thus improves RNA yield. Third, mRNA will preferentially fractionate into the phenol phase under certain conditions (Smith *et al.*, 1970; Lee *et al.*, 1971), but this is also prevented by using chloroform (Penman, 1966; Perry *et al.*, 1972; Palmiter, 1973a). I have observed no effect of adding isoamyl alcohol to the chloroform as specified in some procedures.

For mRNA translation studies, the RNA precipitate was washed twice with 2 ml of 3 M sodium acetate (pH 6) (see Palmiter, 1973a and text). The RNA was then dissolved in 0.1 M sodium acetate (pH 7.0), precipitated overnight with ethanol, and collected by centrifugation; the pellet was dried with N_2 and dissolved in 20 mM Hepes at a concentration of 0.5–1.0 mg/ml. RNA pellets can be washed most effectively, and dissolved more easily, if they are smeared on the inside of the tube with a glass rod before adding the high salt solution, or buffer.

Assay for Ovalbumin mRNA Activity. RNA was isolated and washed as described above. Two samples, one twice the amount as the other and containing between 5 and 40 μg of total polysomal RNA, were assayed essentially as described (Palmiter, 1973a) using lysates prepared from rabbit reticulocytes. The incorporation of [^{3}H]isoleucine into ovalbumin was determined by immunoprecipitation (Rhoads *et al.*, 1971; Palmiter, 1973a). When RNA which has been washed with salt is used, the incorporation of amino acids into ovalbumin is directly proportional to RNA added up to at least 250 μg of RNA/ml of reaction mixture (Palmiter, 1973a; Palmiter and Smith, 1973). Some of the experiments reported were performed with lysates having different endogenous activity; thus, mRNA activities in one experiment cannot be compared with another (see Palimiter, 1974).

Sucrose Gradient Analysis of Polysomes and Ribosomal Subunits. Samples containing 2–10 A_{260} units in up to 1 ml of buffer were layered over 11.5-ml sucrose gradients and centrifuged at 280,000g_{max} using either the SB 283 rotor (International Equipment Co.) or the SW 41 rotor (Beckman); details are given in the figure legends. After centrifugation, the gradients were pumped through a 10-mm flow cell and a continuous recording prepared using either a Gilford, Isco, or Cary spectrophotometer. Some of the optical density scans were obtained using different monitoring devices which accounts for the apparently different levels of resolution. The S values associated with ribosomes and their subunits are approximate; they are used for nominal purposes only.

Acrylamide Gel Electrophoresis. Samples of RNA in 50 μl of electrophoresis buffer were layered over 2% acrylamide–0.5% agarose gels prepared as before (Haines *et al.*, 1974), subjected to electrophoresis for 3 hr at 5 mA per gel, soaked several hours in H_2O, and scanned at 260 nm using a Gilford linear transport device.

Results

Optimal Conditions for Magnesium Precipitation of Ribosomes. The results shown in Figure 1 confirm the original ob-

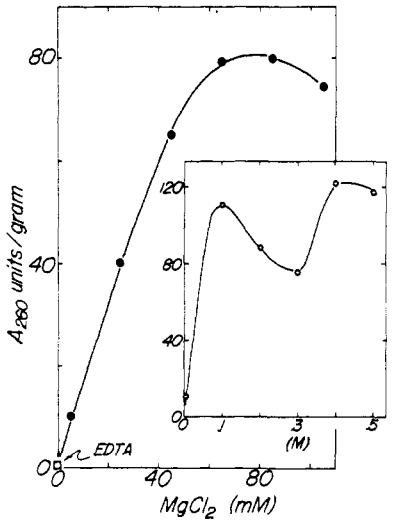


FIGURE 1: Effect of magnesium concentration on ribosome precipitation. A 10% homogenate of laying hen oviduct was prepared in buffer A and centrifuged as described under Methods. Aliquots (2 ml) were diluted 1:1 with modified buffer B so that the final Mg^{2+} concentration was that indicated. One sample was diluted 1:1 with buffer A and EDTA was added to 25 mM (□). After 1 hr at 0°, the samples were layered over 4-ml pads of 0.2PBM, modified to contain the same Mg^{2+} concentration as the sample, and centrifuged for 10 min at 27,000*g*. The pellets were resuspended in 2 ml of 0.5% SDS and the absorbance at 260 nm was determined. The experiment shown in the *inset* was obtained using a different hen oviduct and concentrations of Mg^{2+} up to 0.5 M.

servations of Takanami (1960) that maximum precipitation of ribosomes can be achieved by adding $MgCl_2$ to a tissue homogenate to a final concentration of 60 mM or more. The results are expressed as A_{260} units precipitated/g of oviduct (wet weight); approximately 11 A_{260} units equal 1 mg of ribosomes. That the A_{260} units are indeed in ribosomes is documented below. The 5 mM Mg^{2+} present in the usual polysome buffer (PB) precipitated about 10% of total ribosomes; inclusion of EDTA prevented precipitation entirely (Figure 1). Concentrations of Mg^{2+} up to 500 mM effectively precipitated ribosomes although there was an unexpected decrease in the A_{260} yield at intermediate concentrations (Figure 1, inset). In the experiments which follow, 100 mM Mg^{2+} was generally used to precipitate ribosomes.

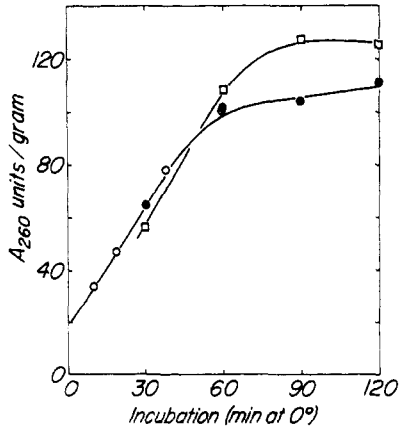


FIGURE 2: Time course of ribosome precipitation with magnesium. A 10% homogenate of laying hen oviduct was prepared and centrifuged as described under Methods. Aliquots (2 ml) were diluted 1:1 with buffer B (○, ●; two separate experiments) or buffer B which was modified so that the final Mg^{2+} concentration was 0.4 M (□). Samples were incubated at 0° for the times indicated and then layered over 4-ml pads of 0.2PBM and centrifuged for 10 min at 27,000*g*. The pellets were suspended in 0.5% SDS and the absorbance at 260 nm was determined.

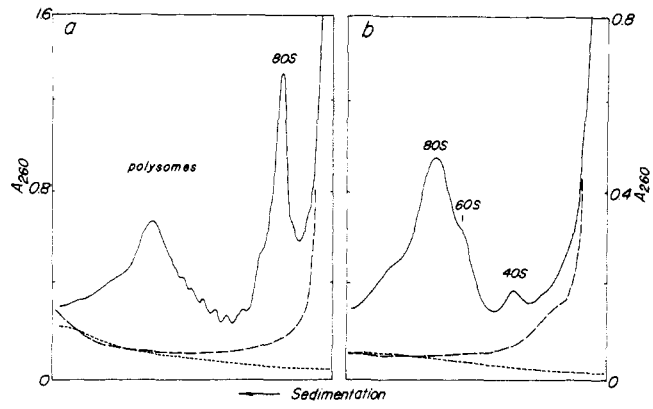


FIGURE 3: Sedimentation profile of polysomes (a) and ribosomal subunits (b). A 10% homogenate of laying hen oviduct was prepared and centrifuged as described under Methods. Portions were diluted 1:1 with either buffer A or B and incubated for 1 hr at 0°, then centrifuged for 10 min at 27,000*g*. Another homogenate was also prepared, diluted 1:1 with buffer A, but not incubated. Aliquots (0.6 ml) of the supernatants were layered over 0.5–1.5PB gradients and centrifuged for 90 min (a), or they were layered over 0.5 to 1.0PB gradients and centrifuged for 6 hr (b). Fresh homogenate, diluted 1:1 with buffer A (———); similar, but slightly lower profiles were obtained with samples which had been incubated for 1 hr (not shown). Homogenate diluted 1:1 with buffer B and incubated 1 hr at 0° (– – –); blank gradient (- - - -).

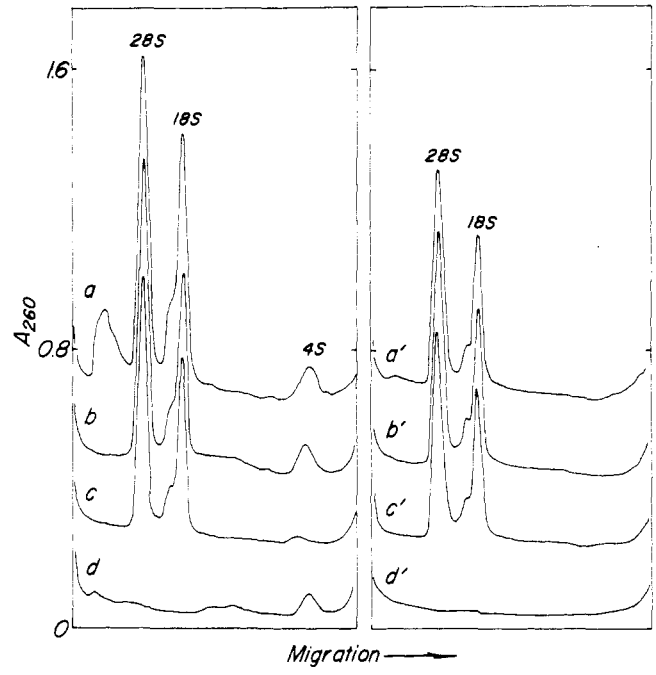
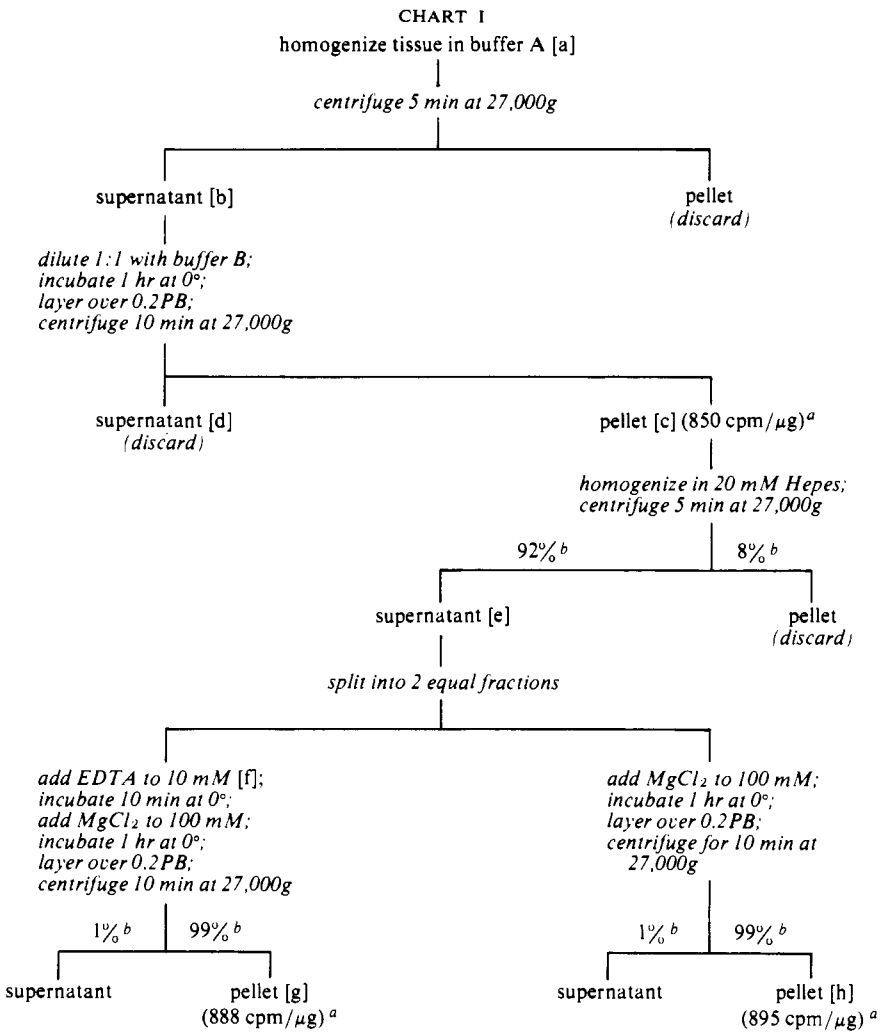


FIGURE 4: Electrophoresis of RNA extracted from oviduct homogenates at various stages in the magnesium precipitation procedure. A 10% homogenate was prepared and the ribosomes were precipitated as described under Methods; the letters a–d refer to the stages designated in Chart I. RNA was extracted as described under Methods and either used directly (a–d) or washed twice with 3 M sodium acetate and then applied to gels a′–d′. Total homogenate (a, a′); homogenate after first centrifugation (b, b′); Mg^{2+} precipitate (c, c′); supernatant remaining after Mg^{2+} precipitation (d, d′).

Maximum ribosome precipitation was obtained after 1 hr of incubation at 0° (Figure 2). This temperature was chosen to minimize RNase activity. The kinetics were similar with either 100 or 400 mM Mg^{2+}. If the 10-min centrifugation time is included in the incubation time, then the curves in Figure 2 ex-

CHART I



[a]Ovalbumin mRNA activity determinations; [^{3}H]isoleucine incorporation/μg of RNA. [b]Percentages refer to recovery of total A_{260} units in each fraction.

trapolate through the origin.

Several factors influence ribosome recovery. Complete homogenization of the tissue is crucial, otherwise substantial losses are incurred during the first centrifugation. Ribosome concentration during the Mg^{2+} precipitation step is also important. In a dilution experiment, recovery was 88–100% when ribosome concentration exceeded 1.6 A_{260} units/ml, but fell to 50% when the sample was diluted to 0.6 A_{260} unit/ml. Thus, if yields less than about 30 A_{260} units/g wet weight are consistently observed, a more concentrated homogenate should be tried. A low monovalent cation concentration is critical for ribosome precipitation. Addition of NaCl or KCl to buffer A reduced ribosome recovery by 75% at 60 mM, by 90% at 100 mM, and completely prevented precipitation at 400 mM. Monovalent ions compete with Mg^{2+} for ribosome binding sites (Petermann and Pavlovec, 1967). Finally, control of RNase is essential (see below).

To establish whether precipitation of ribosomes was quantitative, homogenates were treated with Mg^{2+} as shown in the flow chart (Chart I) and the supernatant remaining after Mg^{2+} precipitation [d] was layered over a sucrose gradient and centrifuged to display the polysomes. Comparison of the A_{260} profile with that of an untreated sample [b] reveals that all polysomes and monosomes were removed by the Mg^{2+} precipitation procedure (Figure 3a). If samples were centrifuged longer in order to display ribosomal subunits, then it is apparent that Mg^{2+} also precipitated 60S and 40S subunits (Figure 3b).

Characterization of the Magnesium Precipitates. To ascertain whether Mg^{2+} precipitates tRNA along with rRNA, RNA was extracted from samples at various stages of the fractionation procedure and subjected to acrylamide gel electrophoresis (Figure 4a–d; the letters correspond to the fractionation stages [a–d] in Chart I). The bulk of the DNA which migrates slower than 28S RNA in Figure 4a was removed during the first centrifugation, leaving primarily rRNA and tRNA (Figure 4b). The amount of rRNA in fractions [a] and [b] was virtually identical, indicating that very little rRNA was lost during the first centrifugation. All the identifiable rRNA was recovered in the Mg^{2+} precipitate (Figure 4c), while the bulk of the tRNA remained in the Mg^{2+} supernatant (Figure 4d).

To determine whether mRNA was precipitated by Mg^{2+}, ovalbumin mRNA activity in ribosomes isolated by Mg^{2+} precipitation was compared to that isolated by standard ultracentrifugation techniques (Palacios *et al.*, 1972). Table I shows that ovalbumin mRNA specific activity (cpm/μg of RNA) was about 30% lower in the Mg^{2+} precipitated RNA (samples E, E′) compared to the controls (samples A–D). However, the yield of RNA was greater, making the total ovalbumin mRNA yield comparable in the two treatments. These results are accounted for by the fact that Mg^{2+} precipitates monosomes as well as polysomes, whereas the ultracentrifugation technique selects against monosomes which constitute 20–30% of total ribosomes in hen oviduct (Figure 3a).

Table II summarizes the results of various resuspension

TABLE I: Comparison of Ovalbumin mRNA Recovery by Ultracentrifugation and by Magnesium Precipitation.[a]

Treatment	Gradient Buffer	RNA Recovery μg/mg wet wt	Ovalbumin mRNA Activity: cpm/μg of RNA	Ovalbumin mRNA Activity: cpm/mg wet wt
Ultracentrifugation (100 min at 280,000g_{max})				
A. no dilution	1.0PB	3.15	1360	4300
B. dilution 1:1 with buffer A	1.0PB	4.05	1320	5350
C. dilution 1:1 with buffer B	1.0PB	4.05	1570	6350
D. dilution 1:1 with buffer B	1.0PBM	4.40	1360	6000
Low speed centrifugation (10 min at 27,000g_{max})				
E. dilution 1:1 with buffer B	0.2PBM	5.75	960	5510
E′. same as E	0.2PBM	6.00	950	5700

[a] A 10% homogenate of hen oviduct was prepared in buffer A and centrifuged as described under Methods. Samples were treated as shown and 6-ml aliquots were either layered over step gradients consisting of 4 ml of 1.0 M sucrose and 1 ml of 2.5 M sucrose and centrifuged in the ultracentrifuge ("cushion method"), or precipitated with Mg^{2+} and the ribosomes collected as described under Methods. RNA was extracted from the bottom 2 ml of the step gradients, or from the Mg^{2+} precipitates which were suspended in 20 mM Hepes as described under Methods. Ovalbumin mRNA activity was assayed using a rabbit reticulocyte lysate (see "Methods").

TABLE II: Resuspension of Magnesium-Precipitated Ribosomes.[a]

Treatment	% Soluble
1. Centrifuge through 0.2PBM, homogenize in PB, dialyse against 40% PB	78
2. Centrifuge through 0.2PBM[b]	
(a) Resuspend in PB	19
(b) Resuspend in 20 mM Hepes	50
3. Centrifuge through 0.2PB[b]	
(a) Resuspend in PB	50
(b) Resuspend in 20 mM Hepes	84–92

[a] Hen oviduct polysomes were precipitated with Mg^{2+} as described under Methods, centrifuged through buffered sucrose, and then homogenized in the buffers indicated. In experiment 1, the sample was dialysed overnight at 0° and then centrifuged 5 min at 27,000g. In 2 and 3, the samples were centrifuged after about 5 min. The percentage of the total A_{260} which remained in the supernatant is indicated. [b] Recovery of total A_{260} in experiments 2 and 3 was identical.

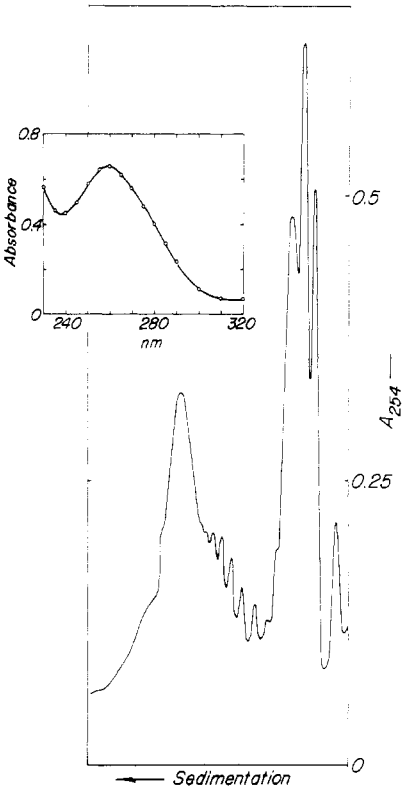


FIGURE 5: Sedimentation profile of resuspended ribosomes. Ribosomes (3.3 A_{260} units) at stage [e] in Chart I were layered over a 0.5–1.5PB gradient and centrifuged for 90 min (see Methods). The absorbance profile of the resuspended ribosomes is shown in the inset.

methods. After dialysis, 78% of the A_{260} units remained in solution (experiment 1); however, an even greater yield could be obtained by centrifuging the Mg^{2+} precipitate through a low Mg^{2+} buffer (PB) and then homogenizing the ribosomes in a buffer without Mg^{2+} (experiment 3b). This procedure resulted in about 90% solubilization in just a few minutes. Control experiments showed that the recovery of ribosomes was identical whether they were centrifuged through buffer PB or PBM.

Figure 5 shows that the polysomes resuspended in Hepes (Table II, experiment 3b) had essentially the same profile as fresh polysomes. Ribosomal subunits were more prominent than hitherto observed in fresh polysomes; they may arise from monosomes due to the low Mg^{2+} concentration.

A typical absorbance spectrum of resuspended ribosomes (Figure 5, inset) reveals 260/280 and 260/235 ratios that are somewhat lower than pure ribosomes (Petermann, 1964). In addition, a high absorbance at 320 nm was observed sometimes which is not characteristic of either RNA or protein. The nature of the contaminants is unknown.

Immunoprecipitation of Ovalbumin-Synthesizing Polysomes. Polysomes resuspended as described in Table II (experiment 3b) can be used for the immunoprecipitation of a specific polysome class. Up to 30% of total hen oviduct ribosomes prepared by these techniques were precipitated with RNase-free, anti-ovalbumin [the curve resembled Figure 6A in Palmiter *et*

TABLE III: Yield of RNA from Various Tissues Using Magnesium Precipitation.

Tissue	Absorbance Ratio 260/280	Yield A_{260} units/g wet wt
Slime mold[a]	1.69	18.2
Silk moth[b] (chorionating follicles)	1.71	30.0
Chicken[c] (brain)	1.55	33.5
Chicken[c] (kidney)	1.78	86
Chicken[c] (liver)	1.78	191
Chicken[c] (oviduct)	1.73	111
Rabbit[d] (reticulocytes)	1.90	89

[a] *Dictyostelium discoidium;* suspension culture in log phase growth (Loomis, 1971); a gift from R. Ziegler and M. Brenner. [b] *Antheraea polyphemus* (Paul *et al.*, 1972). [c] Estrogen-primed immature chicks (Palmiter *et al.*, 1970). [d] Phenylhydrazine-treated rabbits (Palmiter, 1973a).

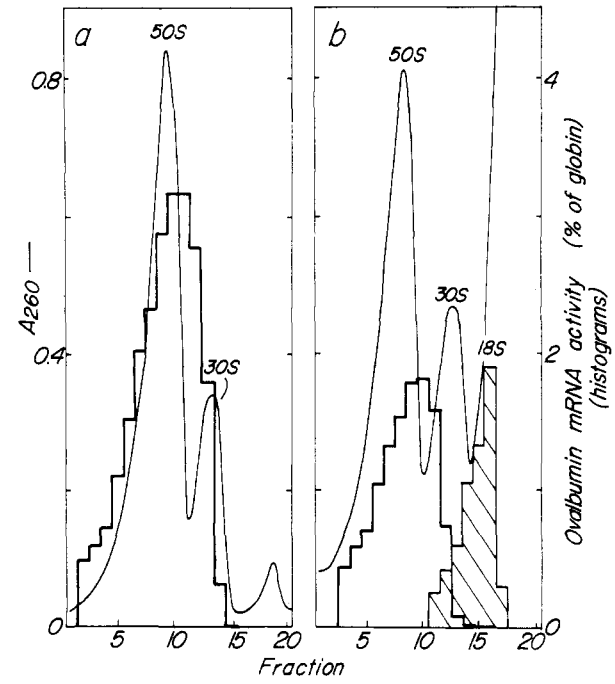


FIGURE 6: Sedimentation of EDTA-derived ribosomal subunits and ovalbumin mRNP particles: (a) EDTA was added to hen oviduct polysomes (1.6 μmol/A_{260} unit); 10 A_{260} units were layered over a 0.5–1.0 M sucrose gradient in 25 mM NaCl–25 mM Tris (pH 7.5); and the sample was centrifuged for 7 hr. (b) Hen polysomal RNA (55 μg) which had been extracted with SDS–phenol–chloroform was added to a rabbit reticulocyte lysate (500 μl total volume) and incubated for 10 min at 26° under standard conditions for protein synthesis except that no label was present. The sample was diluted 1:1 with 40 mM EDTA, layered over a sucrose gradient, and centrifuged as in (a). Another aliquot of hen polysomal RNA (55 μg) was mixed with an equal volume of 40 mM EDTA and layered directly on an identical gradient and centrifuged as in (a). After centrifugation, the A_{260} profiles were obtained by pumping the samples through a 10-mm flow cell and 0.6-ml fractions were collected. Aliquots (50 μl) were assayed directly in reticulocyte lysates for ovalbumin mRNA activity; the incorporation of [^{3}H]isoleucine into ovalbumin is plotted as a percentage of globin synthesis which ranged from 240,000 cpm per 167-μl assay with fractions from the bottom of the tube to 310,000 cpm at the top: (—) absorbance at 260 nm of EDTA-derived ribosomal subunits; (open histograms) ovalbumin mRNA activity from EDTA-derived mRNP particles; (shaded histogram) ovalbumin mRNA activity in hen polysomal RNA shown at ½ scale for clarity (A_{260} trace not shown).

al. (1972)]. The extent of precipitation is comparable to that obtained with ribosomes isolated by ultracentrifugation if the dilution of polysomes by monosomes is taken into account.

Precipitation of mRNP Particles. Essentially all translatable ovalbumin mRNA is located in polysomes (Palmiter, 1973b; Rhoads *et al.*, 1973); thus, it was not possible to determine whether Mg^{2+} precipitates natural mRNP particles. However, it was possible to test whether Mg^{2+} would precipitate mRNP particles derived from polysomes by treatment with EDTA. Figure 6a shows a typical sedimentation profile of EDTA-produced subunits; similar results were obtained with amounts of EDTA ranging from 0.04 to 2 μmol/A_{260} unit of ribosomes. EDTA-derived ovalbumin mRNP particles sedimented as a broad peak with an average sedimentation value of about 40 S, compared to a sedimentation rate of 16–18 S for protein-free ovalbumin mRNA (Figure 6b; see also Haines *et al.*, 1974). Ovalbumin mRNP particles of similar size distribution were produced by adding hen RNA to reticulocyte lysates, indicating that chick oviduct proteins are not essential for the formation of these particles (Figure 6b). The experiment outlined in Chart I shows that all of the ribosomal subunits and ovalbumin mRNP particles were precipitable by Mg^{2+} since the recoveries in samples [g] and [h] were virtually identical and the same as the starting material [c].

Application of Magnesium Precipitation of Ribosomes to Other Tissues. The standard conditions developed for hen oviduct (Chart I) were applied to several other chicken tissues and to cells from other organisms as diverse as slime mold, insect, and mammal. In each case the procedure worked without complication. The yields of ribosomes and the 260/280 absorbance ratios obtained are given in Table III. With reticulocytes it was convenient to add an equal volume of buffer B to a 1:1 lysate; however, it was then necessary to increase the density of the sucrose through which the ribosomes were centrifuged. Up to 1.0 M sucrose can be used without loss of ribosomes and with more ease than the 0.2 M sucrose underlayer used in these studies.

Ribonuclease. The success in isolating active ovalbumin mRNA and undegraded oviduct polysomes depends on controlling RNase activity (Palmiter *et al.*, 1970; Palmiter, 1971, 1973a; Rhoads *et al.*, 1973). In these studies a high concentration of heparin was routinely used for this purpose. Concentrations of 1 mg/ml are required for oviduct in certain physiological states; thus, this amount is now used generally. With other tissues it may be possible to use less, or none at all.

If heparin was omitted from buffer A, less than 10% of chicken oviduct ribosomes were precipitable with Mg^{2+}, although a cloudiness was observed at the interface between the sample and the sucrose pad, indicating that some degree of aggregation occurred. To demonstrate that this was due to an effect of heparin on RNase activity, rather than an indication that heparin participates directly in ribosome precipitation, hen polysomes were prepared following standard procedures to step [e] in Chart I. Aliquots (11 A_{260} units in 1 ml of 10 mM Hepes) were incubated for 10 min at 25° with amounts of RNase A varying in orders of magnitude from 200 μg to 2 ng; then an equal volume of buffer B was added and 1 hr later the recovery of ribosomes was determined as in step [h] of Chart I. Recovery was 87% or greater with 2–20 ng of RNase, but fell to 14% with 200 ng, compared to untreated controls. Thus, with a 20-fold molar excess of ribosomes to RNase, the enzyme severely inhibited precipitability. Furthermore, if oviduct tissue was fractionated to step [b] (Chart I) without using heparin,

TABLE IV: Effect of Magnesium and Heparin on Oviduct RNase Activity.[a]

Heparin (μg/ml)	Magnesium (mM)	[^{32}P]-18S rRNA Precipitated (%)
	2.5	51
	100	100
500	2.5	100
500	100	100

[a] A 10% hen oviduct homogenate was prepared in buffer A, either with or without heparin at 1 mg/ml, and centrifuged as described under Methods. Aliquots of the supernatant (250 μl) were diluted 1:1 with either water or 200 mM $MgCl_2$ and 12.5 μl of [^{32}P]-18S rRNA (70,000 cpm) (a gift from R. Gelinas) was added. Samples were incubated for 20 min at 35°; 100-μl aliquots were removed at 0, 5, 10, and 20 min, precipitated with CCl_3COOH, collected on Whatman GF/C filters and counted. The percentage of the zero-time radioactivity which was precipitable after 20 min is shown.

TABLE V: Effect of Salt-Washing Procedure on Recovery of Ovalbumin mRNA Activity.[a]

Sample	Treatment: Salt	Volume Washes (ml)	Washes (no.)	Total RNA Recovery (%)	Ovalbumin mRNA Activity: cpm/μg	% control
1a	0	0	0	100	1260	100
b	NaOAc (0.1 M)	2	0	96	1180	94
2a	NaOAc (3 M)	1	1	102	1180	94
b	NaOAc (3 M)	2	1	107	1110	88
c	NaOAc (3 M)	4	1	94	1210	96
d	NaOAc (3 M)	2	2	94	1320	104
e	NaOAc (3 M)	2	3	92	1210	96
3a	LiCl (2 M)	2	1	91	1330	106
b	LiCl (2 M)	2	2	96	1250	99

[a] Hen polysomal RNA was centrifuged on a sucrose gradient to separate rRNA and ovalbumin mRNA from heparin and low molecular weight RNA (see Haines *et al.*, 1974). RNA in the 14S–30S region of the gradient was precipitated with 2 volumes of ethanol. Aliquots of the ethanol solution containing 187 μg of RNA were dispensed into separate tubes and treated as indicated. Samples 1a and b are controls; in a, the sample remained in the ethanol solution until assay; in b, the RNA was collected by centrifugation, dissolved in 0.1 M sodium acetate (pH 7.0) and precipitated with ethanol. The RNA in samples 2 and 3 was collected by centrifugation, smeared on the inside of the tube with a glass rod, then the indicated amount of 3 M sodium acetate (pH 6.0) or 2 M LiCl, 50 mM sodium acetate (pH 5.0), was added and the mixture vortexed for about 15 sec at moderate speed with the glass rod inside the tube. After 10–20 min at 0°, the RNA was collected by a 10-min centrifugation at 27,000*g*. This process was repeated as indicated. After the washes, all samples were dissolved in 2 ml of 0.1 M sodium acetate (pH 7.0) and precipitated with 2 volumes of ethanol. The RNA was collected by centrifugation, dissolved in 0.5 ml of 20 mM Hepes; aliquots were used to measure RNA recovery and ovalbumin mRNA activity.

which only takes 10 min, then subsequent addition of heparin up to a concentration of 2 mg/ml resulted in less than 15% ribosome recovery.

Heparin is a sulfated polysaccharide with properties similar enough to RNA for it to act as a competitive inhibitor of RNase (Zollner and Fellig, 1953), and for it to fractionate with RNA during SDS–phenol–chloroform extraction and ethanol precipitation. Small amounts of heparin contaminate ribosomes isolated by either the Mg^{2+} precipitation method or by ultracentrifugation. This provides valuable protection during RNA isolation steps, but becomes a serious problem for subsequent mRNA translation experiments since heparin is a potent inhibitor of ribosome initiation (Waldman and Goldstein, 1973; Palmiter, 1973a).

In addition to precipitating ribosomes, 0.1 M Mg^{2+} helps to inhibit RNase activity. As shown in Table IV, 100 mM Mg^{2+} prevents the solubilization of radioactive RNA by oviduct homogenates. In several previous studies it was shown that intact polysomes could be isolated from tissues containing RNase if the homogenizing medium had an appropriate ionic composition (Verger, 1972; Davies *et al.*, 1972; Gelinas and Kafatos, 1973). Generally a high Mg^{2+} concentration was used (10–50 mM) along with a high concentration of monovalent ion (200–500 mM), which would effectively prevent precipitation of ribosomes. It now seems likely that these media are effective because the Mg^{2+} inhibited RNase activity (Gribnau *et al.*, 1970).

Salt-Wash Treatment of RNA. For translational studies, heparin, DNA, and low molecular weight RNA can be effectively removed from mRNA and rRNA by washing ethanol precipitates with high concentrations of salt (Parish, 1972). Table V shows that polysomal RNA (187 μg) can be washed several times with either 3 M sodium acetate or 2 M LiCl without significant loss of total RNA or ovalbumin mRNA activity. In another experiment, varying amounts of rRNA (ranging from 20 to 400 μg) were washed three times with 1 ml of 3 M sodium acetate or 2 M LiCl to ascertain whether recovery was dependent on the amount of RNA present. The average recovery for all samples was 94% (range 84–100%) and the percentage recovery did not correlate with the amount of RNA. The smallest amounts of RNA (20 and 40 μg) were not visible during the washing procedure. In the experiment described above, the RNA was mixed with 9% heparin. Other experiments have shown that 200 μg of rRNA which was contaminated with up to 1 mg of heparin could be washed three times to remove the heparin with 90% recovery of RNA.

Figures 4a′–d′ show acrylamide gel profiles of RNA which was washed with 3 M sodium acetate. Note in Figure 4a′ that the DNA and 4S RNA peaks were removed by this treatment; in all the other profiles the only difference is the loss of the 4S peak.

rRNA and ovalbumin mRNA were also precipitated from solution by addition of an equal volume of 4 M LiCl. However, the recovery was dependent on the RNA concentration and duration of incubation. After 1 hr at 0°, greater than 90% recov-

ery was achieved with 200 μg of RNA/ml; 80% recovery was obtained with 75 μg of RNA/ml; with an overnight incubation, the same recovery was achieved with 40% less RNA.

Magnesium Precipitation of RNA. Salt-washed polysomal RNA was incubated with 5 or 100 mM Mg^{2+} for 1 hr at 0° and then centrifuged through sucrose as in the isolation of ribosomes. Table VI (experiment A) shows that 100 mM Mg^{2+} precipitated 88% of the rRNA while none was precipitated at the lower Mg^{2+} concentration. The addition of either acidic (ovalbumin) or basic proteins (lysozyme) had only slight effects at 100 mM Mg^{2+}, although a small amount of protein was precipitated as detected by a slight rise in the 260/235 ratio of the RNA in the precipitates (the 260/280 ratios were 2.0 or greater). However, in the presence of 5 mM Mg^{2+}, lysozyme precipitated rRNA (experiment A5), but substantial amounts of lysozyme were also precipitated. In the absence of protein, less than 3% of tRNA was precipitated by either concentration of Mg^{2+}. In the presence of lysozyme, tRNA was also precipitable at 5 mM Mg^{2+}.

Although Mg^{2+} precipitated rRNA under these defined conditions, when labeled rRNA was added during the initial homogenization, its recovery by Mg^{2+} precipitation was only 15–25%, whereas added hen polysomes were recovered with yields of 80–100% as judged by the recovery of ovalbumin mRNA activity.

TABLE VI: Precipitation of RNA by Magnesium and Lysozyme.[a]

Expt	RNA	Protein	$MgCl_2$ (mM)	RNA Precipitated (%)
A1	rRNA (84 μg)	0	5	0
2	rRNA (84 μg)	0	100	88
3	rRNA (84 μg)	Ovalbumin (214 μg)	100	84
4	rRNA (84 μg)	Lysozyme (200 μg)	100	93
5	rRNA (84 μg)	Lysozyme (200 μg)	5	97
B1	tRNA (200 μg)	0	5	0
2	tRNA (200 μg)	0	100	2.2
3	tRNA (200 μg)	Lysozyme (200 μg)	5	46
4	tRNA (200 μg)	Lysozyme (200 μg)	100	3.0

[a] Either salt-washed, hen polysomal RNA or *Escherichia coli* tRNA was incubated for 1 hr in 0.5 ml of PB or PBM plus the indicated proteins. After incubation, the samples were layered over 1 ml of 0.2PB or 0.2PBM and centrifuged for 10 min at 27,000*g*. The supernatants were carefully removed and the pellets were dissolved in 1 ml of 0.5% SDS. The absorbance at 235, 260, and 280 nm was measured. Other aliquots of RNA were dissolved directly in SDS to determine 100% values.

Discussion

The Mg^{2+} precipitation method is an easy and inexpensive means of isolating ribonucleoprotein complexes. The procedure is quantitative as shown by the absence of any identifiable rRNP particles or rRNA in the supernatant remaining after the Mg^{2+} precipitate is removed (Figures 3 and 4). Precipitated ribosomes can be easily resuspended and the polysomes appear to be undegraded. Moreover, ribosomes isolated by this technique are active in protein synthesis (Leytin and Lerman, 1969; Clemens and Pain, 1974). Perhaps the most important applications of this technique will be for those cases where large amounts of ribosomes need to be isolated, or concentrated, and when many samples need to be processed in parallel.

The procedure described here differs from most Mg^{2+} precipitation protocols in one important aspect: the detergent is added before homogenization and centrifugation rather than to the "post mitochondrial supernatant." This has two effects. It improves the recovery of ribosomes, especially from tissues with much endoplasmic reticulum like oviduct and liver. Recovery of oviduct ribosomes and ovalbumin mRNA is increased about tenfold by homogenizing with detergents (Palmiter, 1971; Rhoads *et al.*, 1973); with liver the recovery of ribosomes in the post mitochondrial supernatant is generally only 30–50% of total (Howell *et al.*, 1964; Lewis and Tata, 1973; Clemens and Pain, 1974), although recovery can be improved by thorough homogenization (Blobel and Potter, 1967). The procedure described here has the disadvantageous effect that it lyses membranous organelles which might otherwise be removed during the first centrifugation, thereby increasing the contamination of cytosol with other cellular components, *e.g.*, mitochondrial and nuclear ribonucleoprotein complexes. Furthermore, the lysis of lysosomes liberates nucleases. The latter problem has been overcome by using heparin as a RNase inhibitor. The judicious choice of detergents could prevent the dissolution of the nuclear membrane (Mendecki *et al.*, 1972), but the use of high concentrations of heparin precludes their remaining intact (Arnold *et al.*, 1972; Cook and Aikawa, 1973). If detergents are not used at all, entire microsomes can be precipitated by low concentrations of Ca^{2+} or Mg^{2+} (kamath and Narayan, 1972).

Because the method is both quantitative and nondestructive, it is an excellent starting point for the isolation of mRNA. The fact that undegraded polysomes can be resuspended from Mg^{2+} precipitates provides a useful starting point for the isolation of a specific polysome class, and subsequently specific mRNA, by using immunological techniques (Palmiter *et al.*, 1972; Palacios *et al.*, 1973; Delovitch *et al.*, 1972; Schechter, 1974). Alternatively, bulk RNA can be isolated from the Mg^{2+} precipitates by SDS–phenol–chloroform extraction and then further fractionated by a salt-washing procedure to remove contaminating tRNA and 5S RNA. Hybridization of the RNA with immobilized oligo(dT) or poly(U) can be used to separate rRNA from poly(A) containing mRNA (Aviv and Leder, 1972; Sheldon *et al.*, 1972). (Millipore filters and plain cellulose can also be used, but they are less satisfactory because of substantial contamination of mRNA with rRNA; see Haines *et al.* (1974).) Because the ribosomes can be separated from the bulk of the soluble protein and concentrated by Mg^{2+} precipitation, that allows the phenol extraction of RNA to be performed with much smaller, safer volumes of phenol and with greater recovery of RNA.

Bulk RNA can be used for mRNA translational studies but for optimal translation it is necessary to remove certain contaminants; heparin is one such contaminant, but it is certainly possible that other polysaccarides, low molecular weight RNAs (perhaps double-stranded RNA), or DNA are isolated from

tissues during phenol extraction which inhibit ribosome initiation in the cell-free, protein-synthesizing systems. There are several means of removing these contaminants, including sucrose gradient centrifugation, a preliminary mRNA isolation as described above, or a salt-washing procedure. The salt-washing procedure gives quantitative recovery of total RNA and mRNA activity. It is particularly valuable for processing many samples. For translational studies it is useful to retain the rRNA with the mRNA because it automatically allows a meaningful calculation of mRNA specific activity, *i.e.*, mRNA activity can be expressed relative to rRNA recovery. If a mRNA purification step is used, identical recovery of mRNA is generally a tacit assumption.

It is especially important in physiological studies to estimate the extent of mRNA degradation encountered during the isolation procedures, because extensive and variable degradation can easily lead to misinterpretations. One method of determining the extent of mRNA degradation is to compare direct estimates of translational efficiency with estimates based on the ratio of protein molecules synthesized per mRNA in a cell-free system (Palmiter, 1973a). The procedure described here is about twice as good as that described before, *i.e.*, in the best preparations, approximately 80% of total ovalbumin mRNA is isolated in active form. With this activity, ovalbumin accounts for about 12% of total [^{3}H]isoleucine incorporation in reticulocyte lysates when hen polysomal RNA is added at 100 μg/ml. Another approach that has been used is to measure the recovery of mRNA activity in polysomes added prior to homogenization. Recovery of ovalbumin mRNA activity is routinely 80–100%, demonstrating that RNase is effectively controlled in this procedure. However, if heparin is omitted, less than 10% of oviduct ribosomes are Mg^{2+} precipitable and ovalbumin mRNA activity is negligible.

There is extensive evidence that Mg^{2+} is essential for ribosome function and integrity (Weiss and Morris, 1973); it is bound exclusively to the rRNA with approximately 0.5 mol of Mg^{2+}/mol of RNA phosphate (Edelman *et al.*, 1960; Goldberg, 1966; Choi and Carr, 1967). Some of the rRNA is exposed on the surface of the ribosome (Cotter *et al.*, 1967; Cox and Bonanou, 1969; Pinder and Gratzer, 1972; Dessev and Grancharov, 1973). Since rRNA, but not tRNA, can be precipitated by Mg^{2+} under the same conditions used to precipitate ribosomes, and because ribosome precipitation is prevented by RNase, it seems likely that ribosome precipitation involves an effect of Mg^{2+} on the exposed rRNA. However, the detailed molecular mechanism of the precipitation reaction is unknown. RNA tumor viruses are not precipitated by Mg^{2+} (Attardi and Smith, 1962; Levy and Carter, 1968), presumably because the viral proteins completely encapsulate the RNA in the mature virus.

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Covalent Attachment of Fluorescent Groups to Transfer Ribonucleic Acid. Reactions with 4-Bromomethyl-7-methoxy-2-oxo-2*H*-benzopyran†

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ABSTRACT: Pseudouridine in *Escherichia coli* $tRNA^{fMet}$ and 2-thio-5-(*N*-methylaminomet hyl)uridine in *E. coli* $tRNA^{Glu}$ were specifically modified with the fluorescent compound 4-bromomethyl-7-methoxy-2-oxo-2*H*-benzopyran. In reactions with the homologous aminoacyl-tRNA synthetases the modified tRNAs showed acceptor activities of 30 and 10%, respectively, compared to unmodified tRNA.

The chemical modification of tRNA presents a great challenge to organic chemists. Specificity for a particular base and reaction at only one site in the molecule are required if a useful derivative is to be obtained. Such procedures are important in the purification of tRNA species (Gillam *et al.,* 1968). Modified tRNA molecules have aided structural studies on tRNA by fluorescence spectroscopy (Cantor and Tao, 1971), X-ray crystallography (Kim *et al.,* 1973), or electron spin resonance (Kabat *et al.,* 1970; Hara *et al.,* 1970). In addition, these compounds are invaluable in investigating the relationship of structure to biological function of tRNA (see, *e.g.,* Thiebe and Zachau, 1968). The majority of the chemical modification reactions involve the free amino group of the amino acid in aminoacyl-tRNA or the modified nucleosides in tRNA. Not only is their reactivity often higher than those of the four major mononucleotides, but since they only occur once or twice in a tRNA molecule a site specific reaction is more easily obtained.

Continuing our studies on the chemical modification of tRNA with fluorescent groups, we have further investigated the use of 4-bromomethyl-7-methoxy-2-oxo-2*H*-benzopyran (BMB).[1] This compound was described by Secrist *et. al.* (1971) as a specific reagent for 4-thiouridine. We have successfully modified 4-thiouridine in tRNA with this fluorescent group (Yang and Söll, 1973b). In this communication we show that BMB reacts readily also with pseudouridine and 2-thio-5-(*N*-methylaminomet hyl)uridine. Thus, we were able to modify the latter nucleoside in *Escherichia coli* $tRNA^{Glu}$ and the single pseudouridine residue in *E. coli* $tRNA^{fMet}$.

Materials and Methods

General. Uniformly labeled [^{14}C]methionine and [^{14}C]glutamate with a specific activity of 221 and 197 Ci/mol, respectively, were obtained commercially. Pseudouridine and pseudouridine 3′-phosphate were purchased from Sigma. 4-Bro-

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[1] Abbreviations used are: BMB, 4-bromomethyl-7-methoxy-2-oxo-2*H*-benzopyran; BD-cellulose, benzoylated DEAE-cellulose; Nucleotides and nucleosides are abbreviated according to the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (*Biochemistry 9,* 4022 (1970)). ! denotes 2′,3′-cyclic phosphate; ^{2}Sp*, 2-thio-5-(*N*-methylaminomet hyl)uridine 3′-phosphate. One optical density unit at 260 nm (A_{260} unit) is the amount of material/ml of a solution which produces an absorbance of 1 in a 1-cm light path at 260 nm. ^{2}S*-BMB-tRNA designates tRNA to which a BMB residue is attached covalently through the ^{2}S*. ψ-BMB-tRNA designates tRNA to which a BMB residue is attached covalently through the ψ.