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Pulse-Labeled Ribonucleic Acid Complexes Released by Dissociation of Rat Liver Polysomes*

Se Yong Lee† and George Brawerman†

ABSTRACT: Rat liver polysomes, prepared by Mg precipitation and freed of native subunits by differential centrifugation, were dissociated into subunits by various treatments such as EDTA, urea, and 0.5 M KCl at pH 9. In all cases, the pulse-labeled RNA sedimented as heterogeneous material overlapping the two ribosomal subunits. This sedimentation behavior was not significantly altered in a wide range of KCl concentrations. Different centrifugal fractions derived from EDTA-treated polysomes were recentrifuged after further EDTA treatment. A large portion of the labeled RNA showed unchanged sedimentation characteristics. Sodium dodecyl sulfate treatment of these fractions released labeled RNA with considerably smaller *s* values. The range of *s* values of the released RNA was about the same, regardless of the sedimentation range of the fraction from which it was derived.

On the basis of the behavior of the pulse-labeled RNA, it is proposed that the mRNA of polysomes is firmly bound to large amounts of other material, which could serve to bind it to cellular structures. Some radioactivity was present in rRNA. The two rRNA components in the polysomes appeared to be about equally labeled, in spite of the wide difference in the specific radioactivity of the two free ribosomal subunits in the cytoplasm.

Numerous investigations of the nature of mRNA in eukaryotic cells point to its occurrence as nucleoprotein particles. Pulse-labeled RNA in cytoplasmic extracts has been shown to sediment as heterogeneous material in the range of 20–90 S, although treatment with sodium dodecyl sulfate reduces considerably these sedimentation values (Spirin *et al.*, 1964; Nemer and Infante, 1965; Perry and Kelley, 1966; Henshaw and Loebenstein, 1970). Moreover the labeled material was found to exhibit buoyant density values far lower than those of free RNA. Attempts to release pulse-labeled RNA from polysomes by treatment with EDTA have resulted in the appearance of complexes with characteristics similar to those of the free ribonucleoprotein particles (Perry and Kelley, 1968; Cartouzou *et al.*, 1968; Henshaw, 1968). Thus it has been postulated that mRNA is transferred from nucleus to cytoplasm in the form of nucleoprotein particles, and that these nucleoproteins become

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part of the polysome structure (Spirin, 1966). Other studies, however, have indicated the possibility of artifacts originating during cell disruption. It has been shown that free RNA can form large aggregates with cytoplasmic proteins at low ionic strength (Baltimore and Huang, 1970). It has also been reported that when poly(U) interacts with reticulocyte ribosomes in a cell-free protein-synthesizing system, it appears to be released by EDTA treatment as a complex (Nolan and Arnstein, 1969). It is clear that a correct assessment of the significance of the mRNA complexes released from polysomes will require a better knowledge of the nature of these complexes and of the mechanism of polysome dissociation.

The present study was designed to investigate the nature of the pulse-labeled RNA complexes released by EDTA treatment of rat liver polysomes. Extensively purified preparations were used in order to minimize possible contamination by other pulse-labeled RNA components. In order to verify whether the complexes represent an artifact peculiar to the EDTA treatment, different dissociating agents were tried. All treatments resulted in the same heterogeneous sedimentation pattern for the released pulse-labeled RNA. The latter remained bound in the aggregates under conditions which should lead to dissociation of the complexes formed between free RNA and cytoplasmic proteins as described by Baltimore and Huang (1970). Finally, complexes of different sizes were found to contain RNA of very similar sedimentation characteristics, as if the mRNA molecules were bound to widely varying amounts of other material.

Experimental Section

Preparation of Polysomes. Rat liver cytoplasmic extracts were prepared as described previously (Hadjivassiliou and Brawerman, 1967). Animals weighing approximately 150 g, were fasted overnight, and injected intraperitoneally with 50 μCi of orotic acid-6-^{14}C (Amersham-Searle, Des Plaines, Ill., 60 mCi/mmole), 50 min prior to killing. The livers were removed rapidly, chilled in ice-cold STC (10% sucrose–50 mM Tris-HCl (pH 7.6)–3 mM $CaCl_2$), and disrupted in a volume of STC equivalent to six times the weight of liver. The homogenization was carried out gently in a Teflon-glass homogenizer (Thomas, Philadelphia) at low speed, with five up and down strokes. The homogenate was centrifuged at 17,000*g* for 10 min.

Polysomes were obtained from the supernatant as follows. For each 10 ml of supernatant, 4 ml of 20% Triton X-100 and 3.5 ml of 5% sodium deoxycholate were added. Magnesium acetate was next added to a final concentration of 70 mM to precipitate the polysomes. After 1 hr at 0°, the suspension was centrifuged at 17,000*g* for 10 min. In the subsequent steps, all solutions were supplemented with a high-speed supernatant of liver extract in amounts of 20% (v/v), to inhibit RNase activity (Roth, 1956; Blobel and Potter, 1966). It was prepared by disruption of rat liver in three volumes of STC, centrifugation of the homogenate at 39,000 rpm for 4 hr, and storage at −15° after quick freezing in an acetone–Dry Ice bath. The polysome pellets obtained by Mg precipitation were extracted by homogenization in TKM (50 mM Tris (pH 7.6)–50 mM KCl–1 mM $MgCl_2$) and recentrifugation at 17,000*g*. Four extractions were necessary in order to recover the bulk of the polysomes from the pellets. The volumes used for the successive extractions were 4 ml, 2 ml, 1 ml, and 1 ml per 10 g of liver used as starting material. The combined extracts were layered in 4-ml portions over

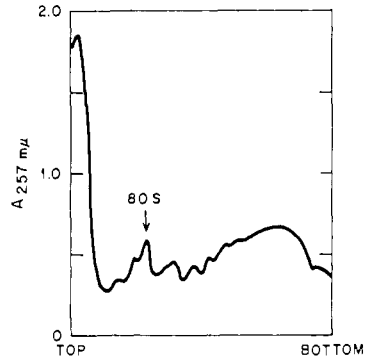

FIGURE 1: Zone sedimentation of polysomes obtained by Mg precipitation. Resuspended Mg pellet (0.05 ml) (see Experimental Section) diluted to 0.1 ml with TKM, layered over 5 ml of a 10–40% sucrose gradient in TKM, centrifuged at 49,000 rpm for 30 min in SW50 Spinco rotor.

5 ml of 20% sucrose in TKM, and centrifuged at 36,000 rpm for 40 min in the Spinco rotor no. 40. The pellets were resuspended in TKM *without liver extract*, and stored in liquid nitrogen.

Zone Centrifugations. Samples were layered over linear sucrose gradients in TKM and centrifuged in Spinco rotor SW50 or SW25. Additional details are listed in the legends of figures. After centrifugation, the solution was pumped from the bottom of the tube through a Uvicord (LKB, Stockholm, Sweden) ultraviolet monitor. For measurements of acid-insoluble radioactivity, fractions were collected and precipitated with ice-cold 10% trichloroacetic acid in the presence of 50 μg of carrier RNA. The precipitates were collected on glass filters, washed with cold 0.1% trichloroacetic acid, dried, and counted in toluene scintillation mix (4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per l. of toluene).

Results

Characteristics of the Polysome Preparations. The use of Mg^{2+} to precipitate polysomes was based on the report by Levy and Carter (1968) that this procedure is effective in separating virus particles from polysomes. The precipitated material consists primarily of heavy aggregates (Figure 1), and appears to have suffered little, if any fragmentation. There seems to be no contamination by ferritin, as judged by the small size of the monomer peak. Glycogen, which contaminates polysomes obtained by ultracentrifugation, was also absent. Polysomes prepared in this manner were highly active in polypeptide synthesis, and showed a greater requirement for soluble factors than those obtained by centrifugation through 2 M sucrose (G. Brawerman, unpublished data). The Mg^{2+} concentration required for precipitation was rather critical. While 70 mM is effective for cytoplasmic extracts in 1% sodium deoxycholate, a lower concentration, 30 mM, must be used when deoxycholate is absent. In the latter case, the polysome precipitate redissolves at Mg^{2+} concentrations above 30 mM.

The material precipitated by Mg^{2+} also contains the free ribosomal subunits. This is clearly indicated by the presence of the large 45S radioactivity peak (Figure 2). Most of this radioactivity is removed when the preparation is centrifuged at 36,000 rpm for 40 min through a 5-ml layer of 20% sucrose. In the present study, two successive centrifugations were

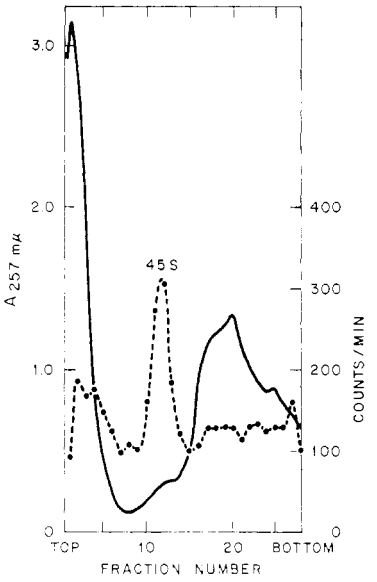

FIGURE 2: Slowly sedimenting components of pulse-labeled, Mg-precipitated polysomes. Resuspended Mg pellet (0.2 ml) layered over a 5-ml 10–30% sucrose gradient in TKM, centrifuged at 49,000 rpm for 90 min in SW50 Spinco rotor. Solid line, recorder tracing of ultraviolet-absorbing material; dashed line with closed circles, acid-insoluble radioactivity.

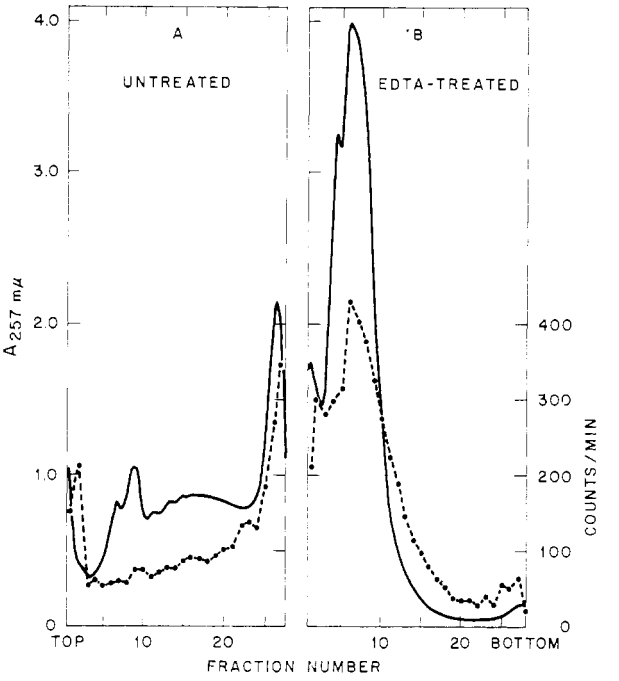

FIGURE 3: Effect of EDTA treatment on sedimentation profile of purified polysomes. Polysome samples (0.1 ml) (7 A_{260} units) in TKM (A) and in TKM with 10 mM EDTA (B) layered over a 4.5-ml linear 10–30% sucrose gradient in TKM; 0.5 ml of 2 M sucrose was placed at bottom of centrifuge tubes to minimize sedimentation of heavy polysomes into pellet. Centrifugation was at 49,000 rpm for 30 min in SW50 Spinco rotor.

carried out to ensure the nearly complete removal of the free subunits (see Figure 5A).

The final polysome preparation still consists primarily of heavy aggregates (Figure 3A). The presence of a significant dimer peak, however, indicates that some fragmentation has taken place in the course of the purification procedure. The radioactivity profile shows an accumulation of label in the heavy fractions. The bulk of this radioactivity is converted into light material after EDTA treatment (Figure 3B). This has been suggested as a criterion for the polysomal nature of the pulse-labeled RNA (Penman *et al.*, 1968). The apparent higher rate of labeling of the more rapidly sedimenting polysomes will be the subject of a separate study.

Dissociation of Polysomes by Various Agents. After EDTA treatment, the pulse-labeled RNA associated with polysomes has been shown to sediment as heterogeneous material overlapping the two ribosomal subunit peaks (Perry and Kelley, 1968; Henshaw, 1968). Similar sedimentation profiles are obtained with the present preparations (Figure 4). The pulse-labeled material appears to be unaffected by KCl concentrations ranging from 0.05 to 0.4 M. Some aggregation of ribosomal subunits is apparent at the latter concentration.

Urea treatment has been shown to convert ribosomes into 40S and 60S subunits still active in protein synthesis (Petermann *et al.*, 1969). A similar treatment developed in this laboratory yields radioactive material with a sedimentation profile analogous to that obtained with EDTA (Figure 5).

Subunits could also be obtained by exposing polysomes to 0.5 M KCl at pH 9.2 in the presence of 1 mM $MgCl_2$. Neither 0.5 M KCl alone nor pH 9.2 alone could produce dissociation. Again the pulse-labeled RNA sedimented as heterogeneous material overlapping both subunit peaks (Figure 5).

The *s* values of the material released by the above treatments are far higher than those of deproteinized pulse-labeled RNA obtained by sodium dodecyl sulfate treatment of the polysomes (Figure 6). This indicates that the heterogeneous material does not represent free RNA. As suggested by Perry and Kelley (1968) and by Henshaw (1968) it may represent ribonucleoprotein complexes. The nature of the material associated with the RNA, however, remains to be determined. The presumed complexes are not due to nonspecific interaction between RNA and proteins liberated during polysome dissociation, since labeled nuclear RNA added to unlabeled polysomes prior to EDTA treatment did not appear in complexes. Similar results have been obtained by Perry and Kelley (1968) and by Henshaw (1968).

Sedimentation Characteristics of the Pulse-Labeled RNA in the Complexes. If the complexes were caused by some nonspecific aggregation during zone centrifugation, it would not be expected that they show the same sedimentation characteristics after an additional EDTA treatment. Fractions from EDTA-treated polysomes were pooled as shown in Figure 7, and recentrifuged after exposure to EDTA. A portion was pretreated with sodium dodecyl sulfate in order to determine the size of the pulse-labeled RNA in each fraction. In the diagrams in Figure 8, a certain portion of the radioactivity represents labeled rRNA. This is clearly evident in 8B, -D, and -E. The amount of rRNA label can be fairly well estimated in these diagrams, and corrections can be made to obtain the sedimentation profiles of the nonribosomal-labeled RNA, as indicated by the dotted lines.

Material lighter than the 30S subunit (fraction A) appears to consist mostly of free RNA, since sodium dodecyl sulfate treatment produces little change in its sedimentation behavior (Figure 8A). It is relatively small (5–14 S), and of high specific activity. In the heavier fractions, a major portion of the pulse-labeled radioactivity shows unchanged sedimentation characteristics (Figure 8). Sodium dodecyl sulfate treatment converts the nonribosomal radioactive material from each of these fractions into species sedimenting mostly between

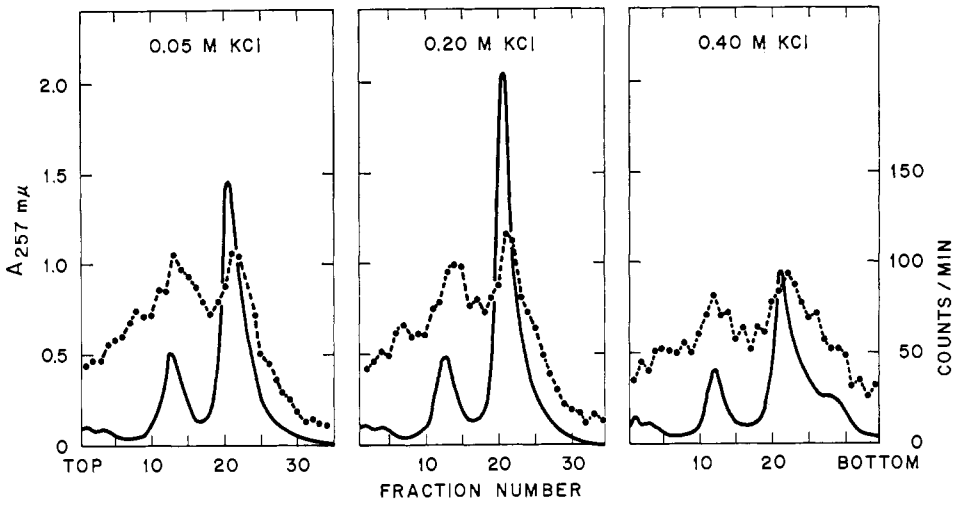

FIGURE 4: Effect of KCl on sedimentation characteristics of pulse-labeled RNA from EDTA-treated polysomes. Purified polysomes, 5 A_{260} units, kept for 15 min at 0° in 0.1 ml of 10 mM EDTA, 25 mM Tris-HCl (pH 7.6), and various KCl concentrations. Samples layered over 5-ml 10–30% sucrose gradients in 5 mM Tris-HCl (pH 7.6) and same KCl concentrations as those used during EDTA treatments. Centrifugation was for 110 min at 49,000 rpm in SW50 Spinco rotor.

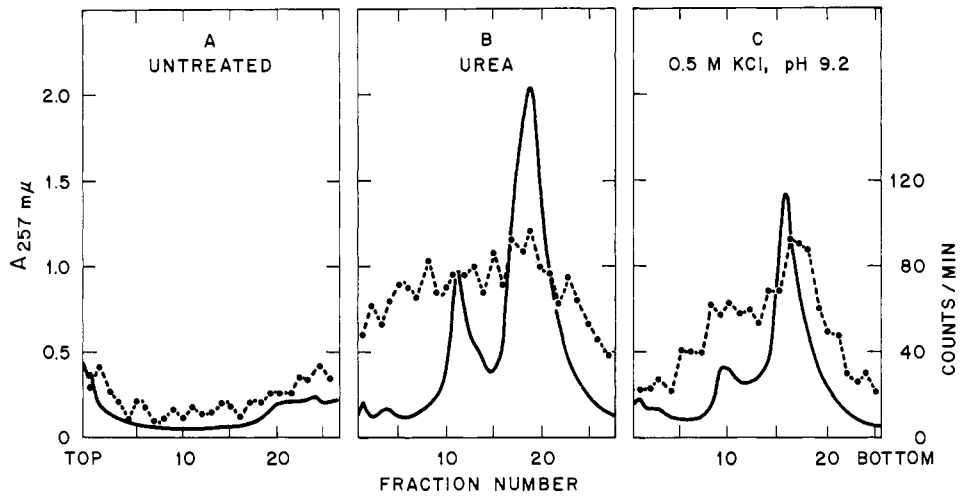

FIGURE 5: Sedimentation characteristics of pulse-labeled RNA after dissociation of polysomes by urea and by 0.5 M KCl at pH 9.2. (A) 3.5 A_{260} units of polysomes in 0.1 ml of TKM containing 5 mM β-mercaptoethanol, layered over 5-ml 10–30% sucrose in TKM with 2.5 mM β-mercaptoethanol. (B) 3.5 A_{260} units of polysomes kept in ice for 10 min in 0.1 ml of TKM, 5 mM β-mercaptoethanol, and 2.4 M urea, then diluted to 0.2 ml with TKM, and layered over sucrose gradient in TKM, 2.5 mM β-mercaptoethanol, and 1.2 M urea. (C) 3 A_{260} units polysomes in 0.1 ml of 50 mM Tris-HCl (pH 9.2), 550 mM KCl, 1 mM $MgCl_2$. 5 mM β-mercaptoethanol, kept in ice for 5 min, then layered over 10–30% sucrose in same concentrations of Tris (pH 9.2), KCl, and $MgCl_2$, and in 1 mM β-mercaptoethanol. Centrifugations at 49,000 rpm for 2 hr in SW50 Spinco rotor.

9 and 21 S. There is a somewhat increased proportion of labeled RNA species with sedimentation values greater than 21 S in the heavier fractions (D, E, and F). It is clear, however, that the apparent size of the complexes is not determined by the size of their RNA component.

Labeling of the Ribosomal Subunits in Polysomes. The sedimentation profiles in Figures 6 and 8 indicate a significant labeling of the rRNA, as should be expected after a 50-min period of orotic acid incorporation (Hiatt, 1962). At this time the free 45S subunit is far more labeled than the 60S subunit (Figure 2). In the polysomes, however, the two rRNA components appear to be about equally labeled. The specific radioactivity of the 28S rRNA component can be determined with reasonable precision from Figure 8D, where most of the heterogeneous pulse-labeled material is separated from the ribosomal peak. Determination of the 18S specific radioactivity from Figure 8B is less precise, because of the considerable overlap by heterogeneous radioactive material. It is clear, however, that the 18S specific radioactivity is not much different from that of the 28S RNA. A rough approximation based on the sedimentation profiles yields values of 70 and 90 cpm per absorbancy unit, respectively, for the large and small ribosomal subunit RNA. Thus in spite of the very different rates of entry of the two newly formed ribosomal subunits into the cytoplasm, these appear to enter into the polysomes at about the same rate.

Discussion

In the present report, the pulse-labeled RNA in the polysome preparations is considered to represent the mRNA associated with the ribosomes. The validity of this assumption

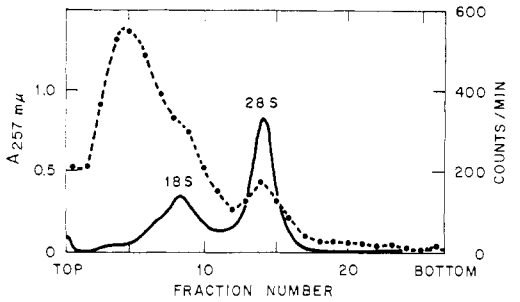

FIGURE 6: Zone sedimentation of pulse-labeled polysomal RNA. Purified polysomes, 7 A_{260} units in 0.3 ml of 0.7% sodium dodecyl sulfate and 10 mM EDTA, layered over a 30-ml 10–30% sucrose gradient in TKM and 1 mM β-mercaptoethanol, centrifuged at 22,000 rpm for 20 hr in SW25 Spinco rotor.

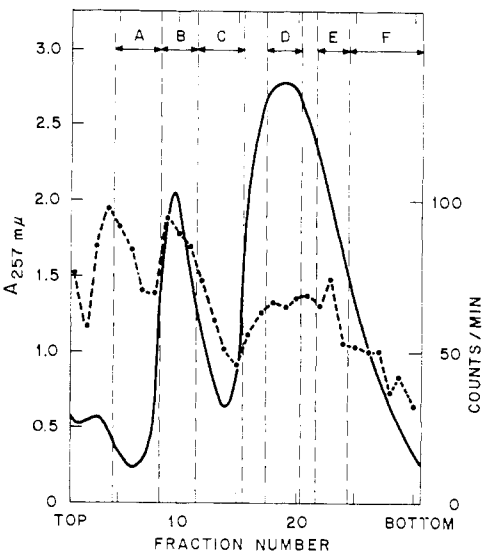

FIGURE 7: Fractionation of EDTA-treated labeled polysomes. Pulse-labeled polysomes, 42 A_{260} units, kept in ice for 15 min in 1.2 ml of 15 mM EDTA (pH 7.0), 50 mM KCl, and 5 mM β-mercaptoethanol. Layered over a 30-ml 5–20% sucrose gradient in TKM and 1 mM β-mercaptoethanol, centrifuged at 20,000 rpm for 12 hr in SW25 Spinco rotor. Samples (0.1 ml) of each fraction used for radioactivity counting; rest pooled as indicated by vertical dashed lines. Solid line, recorder tracing of ultraviolet-absorbing material; dashed line with closed circles, acid-insoluble radioactivity.

depends on the extent of contamination by other pulse-labeled RNA components. Leakage of nuclear RNA during cell disruption, a most serious potential source of contamination, appears to be insignificant with the homogenization procedure used here. This is indicated by the fact that RNA extracted from the cytoplasmic fraction competes only to a very limited extent with nuclear RNA for hybrid formation with DNA (Drews *et al.*, 1968). This has also been verified with RNA from the purified polysomes (S. Y. Lee and G. Brawerman, unpublished data). Leakage of a specific, easily diffusible nuclear RNA species, however, cannot be ruled out. Mitochondria are removed from the cytoplasm by centrifugation prior to detergent treatment. This eliminates RNA associated with these organelles or with other large membrane structures (Attardi and Attardi, 1967). The Mg precipitation procedure may contribute to the elimination of contaminating RNA species. Little is known about its specificity, but at least one type of nucleoprotein, an RNA virus has been shown not to coprecipitate with the polysomes (Levy and Carter, 1968). The two ultracentrifugations through a layer of sucrose effectively remove slowly sedimenting pulse-labeled material with *s* values up to 70 S.

Radioactive rRNA begins to appear in the polysomes after 1-hr labeling, but it represents a relatively small portion of the labeled material, and can be recognized in the experiments described here. The approximately equal labeling of the two rRNA components in the polysomes is rather surprising, in view of the highly unequal labeling of the free ribosomal subunits. A separate study of the ribonuclease-resistant components of polysomes has also indicated that the two rRNA components are equally labeled (D. E. Kelly, S. Y. Lee, and G. Brawerman, in preparation). Studies with ascites cells have shown that the newly formed 45S subunit is not readily incorporated into the polysomes (Hogan and Korner, 1968). The present study suggests that in rat liver the 45S subunit undergoes a maturation process in the cytoplasm about as long as that of the 60S subunit in the nucleus.

The results in this study as well as those previously reported by Perry and Kelley (1968), Cartouzou *et al.* (1968), and Henshaw (1968) indicate that the presumed mRNA is released from polysomes in association with other material. The crucial question to be resolved is whether such complexes preexist in the polysome structure, or are formed during polysome disruption. Baltimore and Huang (1970) have reported that complexes can be formed between free RNA and cytoplasmic proteins. These artificial complexes, however, were shown to be unstable at high ionic strength, while the structures described here do not dissociate in the presence of 0.5 M KCl. Moreover, it is shown by us as well as by others (Perry and Kelley, 1968; Henshaw, 1968) that labeled RNA added to unlabeled polysomes does not appear in complexes after EDTA treatment. It is unlikely that the complexes are due to nonspecific aggregates formed during sedimentation, since much of the labeled material from defined sedimentation zones is shown to resediment with the same sedimentation values after additional EDTA treatment. It can also be stated that the appearance of pulse-labeled RNA in complexes is not due to some peculiarity of the EDTA treatment. The present results show that other dissociating agents, such as high KCl at pH 9.2, or urea, also fail to release free labeled RNA from the polysomes.

While it appears unlikely that the released mRNA complexes result from the interaction of RNA with proteins subsequent to polysome dissociation, the possibility remains that some ribosomal proteins are split off together with the mRNA. Results obtained in a study of RNase-resistant components of rat liver polysomes (D. E. Kelly, S. Y. Lee, and G. Brawerman, in preparation) provide some evidence against this possibility. It was found that pulse-labeled RNA fragments protected from degradation because of their association with ribosomes in the polysome structure, are released free of proteins by subsequent EDTA treatment of the ribosomes. It is not certain, however, that ribosomes previously subjected to RNase respond to EDTA in a manner strictly comparable to that of intact polysomes. In a separate study with the use of mouse Sarcoma 180 ascites cells (Lee *et al.*, 1971), it was also found that mRNA released from polysomes by *in vivo* ribosome runoff appears in complexes with characteristics similar to those described here.

The nature of the presumed mRNA complexes can only be the subject of speculation at the present. The low buoyant density values reported for these complexes have been inter-

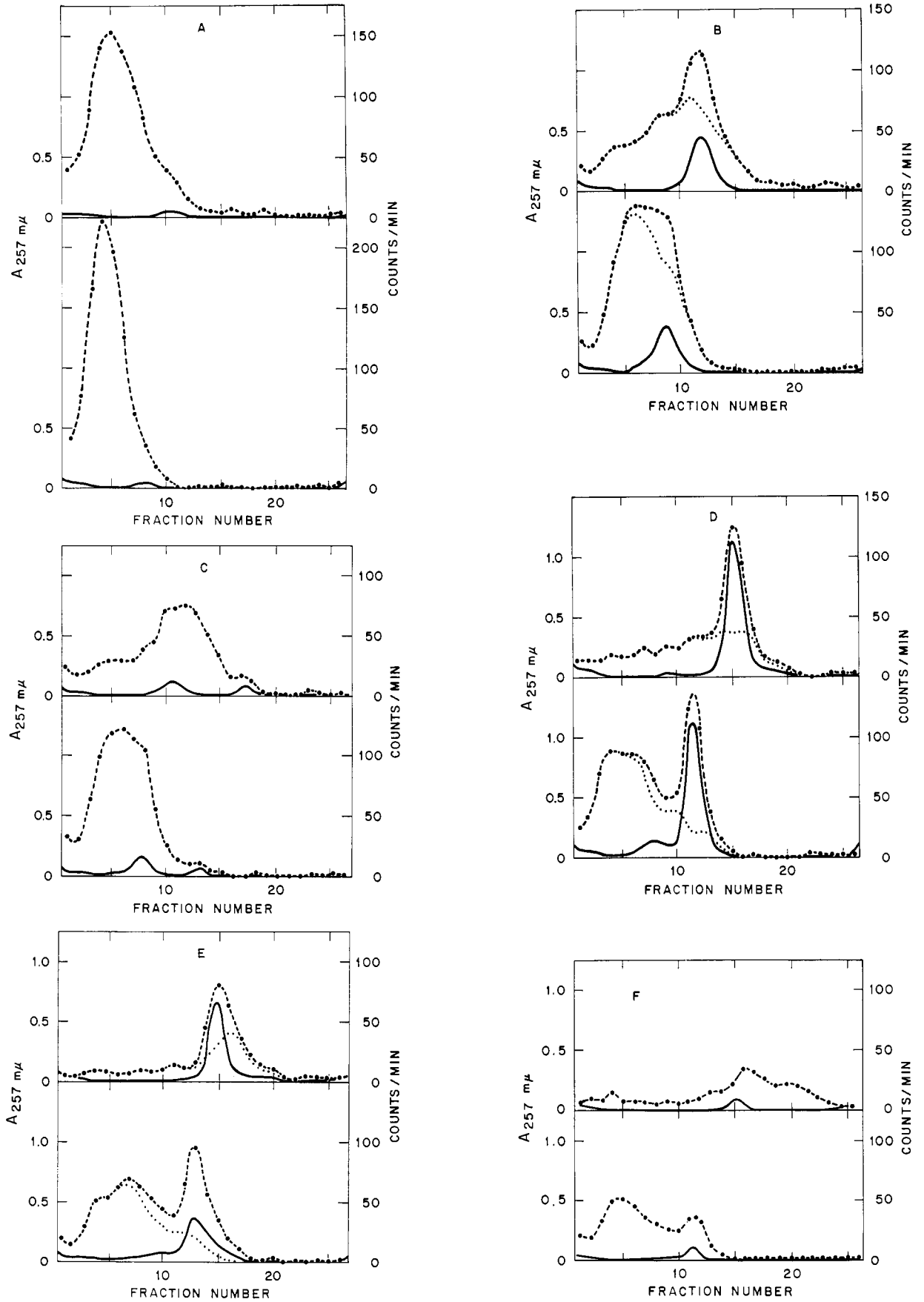

FIGURE 8: Sedimentation characteristics of fractions from EDTA-treated polysomes and of RNA from these fractions. Fractions pooled as shown in Figure 8 were exposed to 10 mM EDTA (upper graphs), and to 0.5% sodium dodecyl sulfate in 10 mM EDTA (lower graphs). They were then centrifuged at 22,000 rpm in SW25 Spinco rotor in 30-ml sucrose gradients in TKM and 1 mM β-mercaptoethanol. (A) 1.5-ml samples centrifuged in 10–30% gradients for 20 hr; (B) 1.2-ml samples, as in part A; (C) 1.3-ml samples, 24 hr, 15–45% gradients; (D) 1.3-ml samples, 20 hr, 15–40% gradients; (E) 1.0-ml sample, 20 hr for upper graph, 1.3-ml sample, 24 hr for lower graph, 15–40% gradients; (F) 1.0-ml sample, as in part D. Solid lines, recorder tracing of ultraviolet-absorbing material; dashed lines with closed circles, acid-insoluble radioactivity; dotted lines, acid-insoluble radioactivity estimated by substracting rRNA labeling (see text).

preted as indicating the presence of large amounts of proteins. The considerable reduction in sedimentation values after SDS treatment also indicates that the RNA is associated with large amounts of other material. The proportion of non-nucleic acid material must be quite variable, since the RNA released from complexes of various sizes shows a nearly constant range of sedimentation values. This is in accord with the relatively wide range of buoyant density values for the complexes described by Henshaw (1968). The RNA appears to be firmly bound in the complexes, since it is not released by 0.5 M KCl (pH 9.2) or by 2.4 M urea. The occurrence of mRNA tightly bound to other cytoplasmic components has been suggested by previous findings in this laboratory. While phenol extraction at neutral pH readily released the rRNA components in the aqueous phase, a rapidly labeled fraction with a high capacity for stimulating polypeptide synthesis by *Escherichia coli* ribosomes could only be extracted at pH 8.3 (Hadjivassiliou and Brawerman, 1967).

The complexes described in this report may provide a clue to the structure of polysome. It is generally assumed that polysomes consist of an mRNA strand with ribosomes in the process of translation spread throughout the length of the mRNA. If large quantities of additional components were also present, they would have to be localized on a region of the mRNA not concerned with translation, in order to leave the decoding portion accessible to ribosomes. Such an arrangement would occur if the functional mRNA were normally attached to other cellular structures at one of its extremities. EDTA treatment would then release the ribosomes and leave the mRNA still associated with remnants of these structures. Such structures could consist of large protein aggregates serving to bind mRNA to cytoplasmic membranes.

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