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Dissociation of Mammalian Polyribosomes into Subunits by Puromycin

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ABSTRACT **Hepatic ribosomes have been dissociated into biologically active subunits as follows. Polysomes were treated at 0°C with puromycin at high ionic strength. This released most of the nascent polypeptide chains without dissociating the polysomes, which retained the mRNA and the tRNA moiety of peptidyl tRNA, but were unable to continue the translation of mRNA. The polysomes were then heated to 37°C, when they dissociated completely into subunits. Similar treatment without puromycin resulted in only partial dissociation.**

A large array of components interact in the polysome during the synthesis of a polypeptide chain and many of these interactions are critically dependent on magnesium ion concentration. Therefore, in the past, magnesium-chelating agents such as EDTA have been used in most attempts to disassemble the eukaryotic polysome in order to isolate and characterize its components. From the mixture of EDTA-dissociated components the two ribosomal subunits were readily isolated, but they turned out to be biologically inactive, i.e., they did not support polyphenylalanine synthesis in a poly(U)-directed system. Recently, several procedures have been published for the dissociation of mammalian polysomes into biologically active ribosomal subunits (1–5). These methods require previous *in vitro* amino acid incorporation with (1, 2) or without puromycin (1, 3), prolonged dialysis against a series of buffer solutions (4), or passage of the polysomes through ion-exchange columns (5).

In this communication we describe a simple procedure for the dissociation of rat-liver polysomes into biologically active subunits, which does not require previous amino acid incorporation *in vitro* and relies only on the complete release of nascent chains by puromycin at high ionic strength at 0°C, followed by a short incubation at 37°C. Preliminary results show that it is applicable to ribosomes from many other sources.

MATERIALS AND METHODS

Fractionation of liver cells

Rats were guillotined without anesthesia and their livers were quickly removed and chilled in several volumes of ice-cold 0.25 M sucrose–TKM [TKM is 50 mM Tris·HCl (pH 7.5 at 25°C)–25 mM KCl–5mM $MgCl_2$]. All subsequent operations were performed in the cold (2–4°C). The livers were minced with scissors and homogenized in 2 vol of 0.25 M sucrose–TKM in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle. A postmitochondrial supernatant fraction (S-17) was prepared by centrifuging the homogenate for 10 min at 17,000 × *g* in the no. 40 rotor of a Spinco model L centrifuge.

Abbreviation: TKM, 50 mM Tris·HCl (pH 7.5 at 25°C)–25 mM KCl–5 mM $MgCl_2$.

Preparation of free polysomes

The procedure has been described (6). In brief, S-17 was layered over a discontinuous sucrose gradient with 3 ml of 2.0 M sucrose–TKM at the bottom. Centrifugation in a Spinco 40 rotor at 40,000 rpm for 24 hr yielded a free polysome pellet, which was rinsed with water and stored at −20°C.

Preparation of supernatant fraction

10 ml of S-17 was centrifuged for 4 hr in a Spinco 40 rotor at 40,000 rpm to obtain a high-speed supernatant (S-105), from which a Sephadex G-50 fraction was prepared by the procedure previously used for the Sephadex G-100 fraction (7) except that the column from which S-105 was eluted was equilibrated with the buffer of the incorporation assay, i.e., 100 mM KCl–50 mM Tris·HCl (pH 7.5 at 25°C)–5 mM $MgCl_2$–1 mM dithiothreitol.

Amino acid incorporation *in vitro*

Endogenous incorporation: The incorporation mixture contained in 1 ml: 0.4 ml of polysomes (1.6 mg/ml); 0.3 ml of Sephadex G-50 fraction; 50 μl of [^{3}H]L-leucine (50 μCi/ml); 25 μl of a mixture of 20 amino acids except leucine (ref. 8); 0.5 μmol of GTP; 1.0 μmol of ATP; 10 μmol of phosphoenolpyruvic acid; 5 μl of pyruvate kinase (EC 2.7.1.40; ref. 6); 100 μmol of KCl; 50 μmol of Tris·HCl (pH 7.5 at 25°C); 5 μmol of $MgCl_2$; and 1 μmol of dithiothreitol.

Incorporation with poly(U): The incorporation mixture was as above, except that the amount of $MgCl_2$ was raised to 15 μmol, and 50 μl of [^{14}C]L-phenylalanine (5 μCi/ml) and 100 μg of poly(U) were added and the amino acid mixture was omitted.

Incubation and measurement of radioactivity were as previously described (7). Counting efficiency for ^{3}H and ^{14}C was 15 and 60%, respectively.

In vivo labeling

For the labeling of nascent polypeptide chains *in vivo*, ether-anesthesized rats received, by injection into the portal vein, 0.2 ml of a neutral solution containing 200 μCi of [^{3}H]L-leucine. After 2 min, the livers were excised and homogenized in 0.25 M sucrose–TKM for cell fractionation and isolation of free polysomes (see above). In order to obtain maximal labeling of tRNA, we injected rats intraperitoneally with 100 μCi of [^{3}H]orotic acid; they were killed after 40 hr (9). A high-

speed supernatant was prepared and used as a source of labeled tRNA.

Assay for the release of nascent labeled polypeptides

Polysomes labeled *in vivo* were suspended in ice-cold water (50.0 OD_{260} units/ml); to 0.2 ml of this suspension, 0.25 ml of an ice-cold compensating salt buffer solution and 0.05 ml of 0.01 M puromycin, pH 7.0, were added. The final concentrations were 0.05 M for the buffer (triethanolamine·HCl, pH 7.5 at 25°C) and 5 mM for $MgCl_2$; the KCl concentration varied from 0 to 1.0 M. After incubation in an ice bath for 15 min, 1.0 M $MgCl_2$ was added to a final concentration of 50 mM (to obtain complete recovery of the ribosomes at the next centrifugation) and the solution was centrifuged for 1 hr at 2°C in the Spinco 40 rotor at 40,000 rpm. The supernate was removed by aspiration as completely as possible and the pellets were dissolved overnight in 0.4 ml of water. Duplicate 0.1-ml aliquots of the solution were pipetted on to Whatman 3 MM filter-paper discs and processed and counted as previously described (7). Other aliquots were diluted with water for determination of the absorbance at 260 nm. Ribosome recovery in the pellet was 100 ± 5%.

Sucrose density gradient analysis

Linear sucrose gradients (12.4 ml) were prepared and centrifuged at 40,000 rpm in the SB 283 rotor of the IEC centrifuge. The concentration of sucrose and of ions in gradients (in addition to other ions, all gradients contained 50 mM triethanolamine·HCl, pH 7.5 at 25°C), temperature, and duration of centrifugation are indicated in the figure legends. The absorbance in the gradient was monitored at 254 nm with an ISCO model D fractionator and UV analyzer. In all figures the direction of sedimentation is from right to left. Positions of subunits, small and large, and of monomers and dimers are marked with *S* and *L*, *1* and *2*, respectively.

Source of materials

The source of animals and chemicals was as described (6). [5-^{3}H]orotic acid (about 1000 Ci/mol) and [^{14}C]L-phenylalanine (375 Ci/mol) were obtained from New England Nuclear, Boston, Mass. Polyuridylic acid was obtained from Miles Laboratories, Elkhart, Ind.

RESULTS AND DISCUSSION

Puromycin has been used in many experiments to release nascent polypeptides from ribosomes, but the reported efficiency varied widely. Recently it was found that the extent of release was a function of monovalent ion concentration and of pH (10, 11) and was optimized in the presence of GTP and supernatant factors (10).

Fig. 1 shows that puromycin at 0°C released less than 10% of the nascent chains in the absence of KCl, when the only monovalent ions added were those of the buffer. Release was enhanced when KCl concentrations were increased up to 1000 mM, when about 80% of the labeled chains came off the ribosomes. Chain release was strictly dependent on puromycin, since in its absence incubation at high ionic strength had no effect. Furthermore, the release occurred in the absence of added supernatant factors or GTP. A more detailed report of the effects of ions on the release of nascent chains, in particular the ratio of monovalent ions to magnesium, puromycin concentration, and temperature variations, will be published elsewhere. We should stress that similar ion effects were found

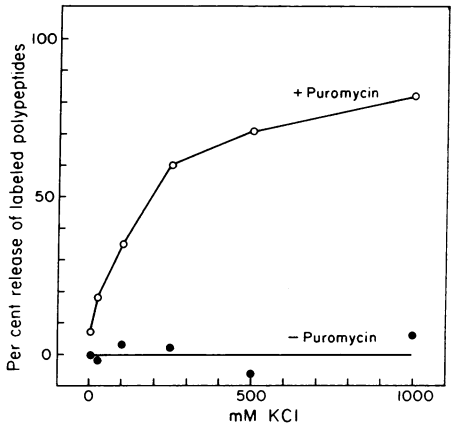

FIG. 1. Effect of KCl concentration on the puromycin-induced release of *in vivo* labeled polypeptides from polysomes. Percent release was calculated as follows. Since ribosome-bound radioactivity in the absence of puromycin after incubation at 0°C can be seen to vary little at the various KCl concentrations (●——●), an average of these samples was taken as 0% release and was used as reference for the calculation of all other points.

when we investigated the coupling of *labeled* puromycin to unlabeled chains. These results ruled out the possibility that the *formation* of peptidylpuromycin proceeded to completion at low ionic strength (at which the chain supposedly remains ribosome-bound) and that only the *release* from the ribosome required high ionic strength.

The data in Table 1 and Fig. 2 demonstrate that the release of a majority of nascent chains at 0°C does not cause the concurrent release of the tRNA moiety of peptidyl-tRNA or of mRNA from the polysomes.

The data in Table 1 were obtained by labeling the peptidyl-tRNA of free polysomes *in vitro* under conditions of amino acid incorporation either with [^{3}H]tRNA or with [^{14}C]phenylalanine. It can be seen that although puromycin at 0°C caused the release of about 70% of the nascent chains under the ionic conditions used (see also Fig. 1), the concurrent release of tRNA amounted to less than 5%. These data are in agreement with results obtained with *E. coli* ribosomes; it was also demonstrated that the release of the tRNA moiety of peptidyl-tRNA is not coincidental with the release of peptidylpuromycin (12).

Fig. 2 (*A*,*B*) shows that the sedimentation profile of polysomes was hardly affected by the release of about 70% of the chains by puromycin at 0°C (see Fig. 1 and Table 1). We concluded that mRNA was not concomitantly released from the polysome complex, since otherwise the appearance of a large amount of monomers or subunits would have been detected. However, the selective removal of the majority of nascent chains, without concomitant release of either mRNA or the tRNA moiety of peptidyl-tRNA, affects the amino acid incorporation activity of the polysomes. Fig. 3 shows that after the release of 70% of the nascent chains from polysomes, the amino acid incorporation activity dropped by more than 80%. It appears that those polysomal ribosomes that had lost their chain were unable to read the rest of the mRNA. These results differ from those reported in (13), where chainless polysomes isolated after incubation of intact rabbit reticulocytes with puromycin were shown to read the rest of the mRNA.

The most striking effect of the removal of nascent chains

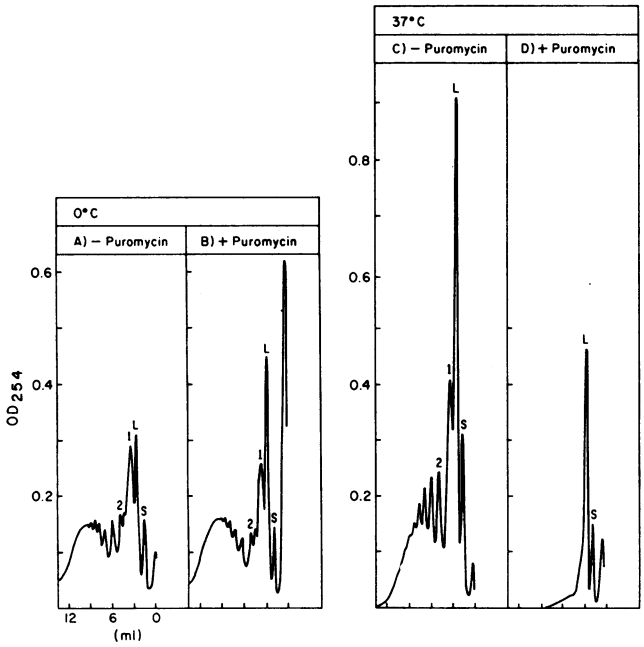

FIG. 2. Sedimentation profiles of polysomes: effect of high ionic strength, temperature, and puromycin. Polysomes (50.0 OD_{260} units/ml) were incubated for 15 min at 0°C, in 500 mM KCl–5 mM $MgCl_2$–50 mM triethanolamine·HCl in the absence (*A*, *C*) or presence (*B*, *D*) of puromycin. After 10 min of additional incubation at 0°C (*A*, *B*) or 37°C (*C*, *D*), the polysomes were fixed with formaldehyde at a final concentration of about 4% and analyzed on 10–40% sucrose gradients in 500 mM KCl, 5 mM $MgCl_2$, and buffer. 100-μl aliquots were layered on gradients *A*, *B*, and *C*, and a 20-μl aliquot on *D*, representing about 2.5 and 0.5 OD_{260} unit of ribosomes, respectively. All gradients were centrifuged for 100 min at 2–4°C.

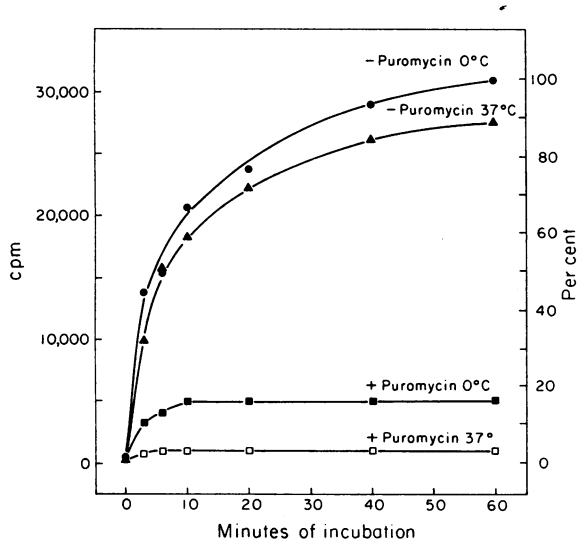

FIG. 3. Endogenous incorporation activity of polysomes after treatment at 0 or 37°C at high ionic strength in the presence or absence of puromycin. Polysomes were incubated as described under Fig. 2 but were not sedimented. Instead they and control polysomes (not incubated) were passed at 2–4°C over a Sephadex G-150 column, equilibrated with 100 mM KCl–50 mM Tris·HCl (pH 7.5)–5 mM $MgCl_2$, to remove puromycin and to lower the ionic strength.

TABLE 1. *Differential release of the peptide moiety and the tRNA moiety of peptidyl-tRNA from polysomes*

Puromycin	Temperature of incubation, °C	[³H]tRNA cpm/mg ribosomes	[³H]tRNA % release	[¹⁴C]Peptide cpm/mg ribosomes	[¹⁴C]Peptide % release
−	0	224	0	2,680	0
+	0	217	3	860	68
+	37	51	78	650	76

Free polysomes were labeled *in vitro* in an amino acid incorporation system for 2 min as in *Methods*, except that one aliquot of the incorporation mixture contained 1.0 μCi of [¹⁴C]-phenylalanine to label the nascent chains, whereas the other contained 0.3 ml of a Sephadex G-50 fraction derived from a high-speed supernate after *in vivo* labeling with [³H]orotic acid to label the tRNA portion of the peptidyl-tRNA. The ribosomes were recovered separately from both samples and were assayed separately for release of [¹⁴C]peptides and [³H]tRNA, at 500 mM KCl, 50 mM buffer, and 5 mM $MgCl_2$ as described in *Methods*, except that ribosomes were centrifuged without the addition of 1.0 M $MgCl_2$. The pellets were resuspended in water, and aliquots were taken for the determination of RNA (9) and radioactivity insoluble in cold acid (RNA) or hot acid (peptides).

from polysomes at 0°C was observed when they were heated to 37°C. Fig. 2*D* shows an apparently complete dissociation of these polysomes into subunits, whereas only a partial dissociation was observed with polysomes that had not been treated with puromycin (Fig. 2*C*). This partial dissociation was found to involve ribosomes that had no nascent chains as well as some chain-bearing ribosomes which, under the conditions used, dissociated as subunits from the polysome complex.

Heating the chainless polysomes to 37°C also caused the release of the tRNA moiety of peptidyl-tRNA (Table 1). The ribosomal subunits isolated from the subunits from polysomes were found to contain no nascent chains and no ap-

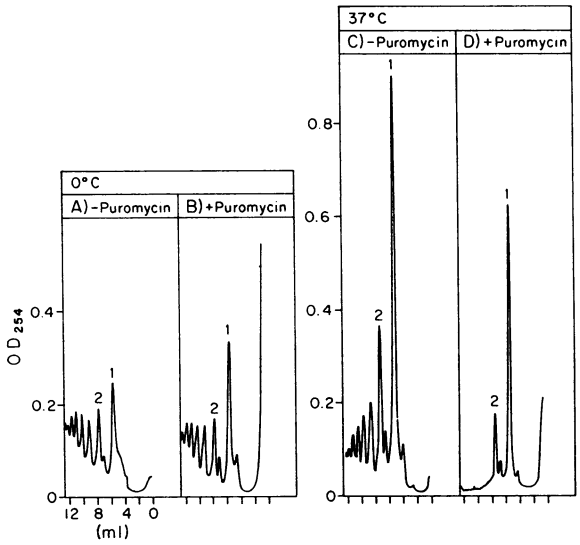

FIG. 4. Same experimental conditions as in Fig. 2, except that formaldehyde treatment was omitted and the sucrose gradients had low ionic strength (25 mM KCl–5 mM $MgCl_2$–buffer).

preciable amounts of tRNA (to be published). Thus, the apparently incomplete release of these molecules observed in Table 1 and Fig. 1 may result from their cosedimentation with ribosomal particles during differential centrifugation. In Fig. 1, for instance, all the chains may have reacted with puromycin at high ionic strength and been detached from the large subunit but, because of the relative insolubility of some of the resulting peptidylpuromycins, they would cosediment with the ribosomes and would be recorded as unreleased.

It should be noted that the sedimentation profiles shown in Fig. 2 were obtained after a brief formaldehyde fixation to inhibit any dissociation of the polysome complex or reassociation of its dissociated components that might occur during sucrose gradient centrifugation under various ionic conditions. Fixation in formaldehyde preserves the state of aggregation of ribosomes and immediately prevents (data not shown) the dissociation of the polysome complex by magnesium-chelating agents (EDTA added to polysomes within 5 sec after the addition of formaldehyde did not dissociate the polysomes).

A dramatic example of reassociation occurring during sucrose gradient centrifugation of unfixed ribosomes can be seen in Fig. 4. Samples identical to those in Fig. 2 were used, except that formaldehyde fixation was omitted and sucrose gradients contained lower concentrations of ions. The two ribosomal subunits seen fully dissociated in Fig. 2*D* reassociated completely into ribosomal monomers and dimers (Fig. 4*D*); reconstitution into polysomes or dimerization of small or large subunits was not detected. Furthermore, the sedimentation profiles of Fig. 4*A* and *B* confirm the results obtained with the formaldehyde technique in Fig. 2*A* and *B*, namely, that the release of nascent chains at 0°C leaves the polysome intact.

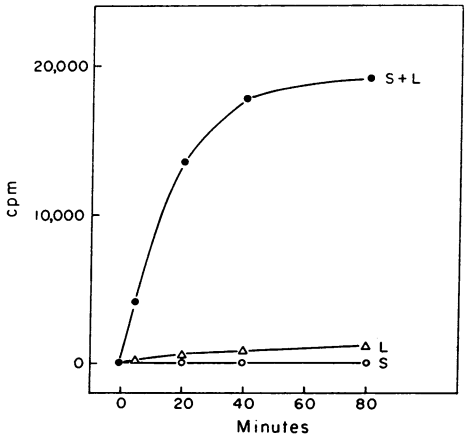

FIG. 5. Poly(U)-directed polyphenylalanine synthesis with isolated ribosomal subunits. Ribosomal subunits were prepared as described under Fig. 6, except that the $MgCl_2$ concentration during the puromycin reaction was 1.5 mM and the amount layered on each sucrose gradient was increased to 0.5 ml. Since the two subunit peaks were well separated from each other on the sucrose gradients, each peak was collected completely with its ascending and its descending part. The subunits were sedimented and resuspended in water to a concentration of 250 μg/ml for the small subunit and 750 μg/ml for the large subunit. Incorporation mixtures (1 ml) contained (*1*) 0.2 ml of small subunit (S); (*2*) 0.2 ml of large subunit (L); (*3*) 0.2 ml of small subunit and 0.2 ml of large subunit (S + L); (*4*) as (*3*), but without poly(U); (*5*) no ribosomal subunits. Only the incorporation kinetics of *1*, *2*, and *3* are shown. *4* and *5* showed no incorporation above background, which was equivalent to the incorporation by the small subunit alone.

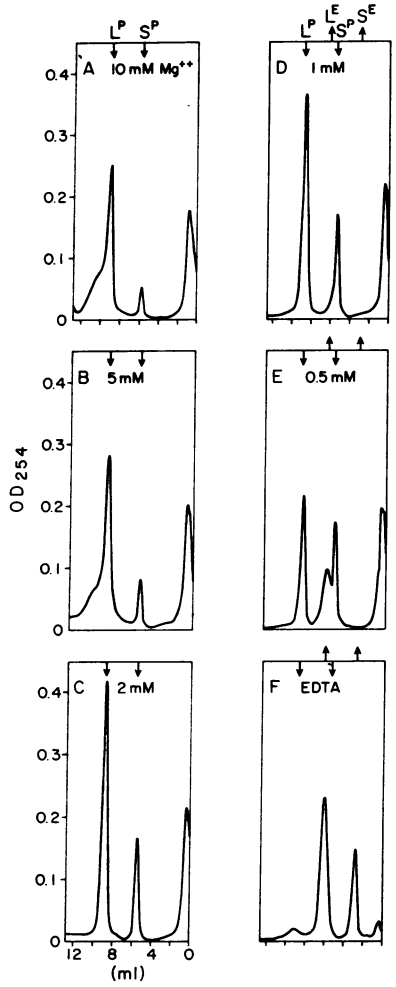

FIG. 6. Effect of magnesium concentration on the dissociation of polysomes into subunits by treatment with puromycin at high ionic strength. Comparison with EDTA subunits.

Polysomes (50.0 OD_{260} units/ml) were incubated for 15 min at 0°C followed by 10 min at 37°C in 500 mM KCl, buffer, pyromycin, and magnesium concentrations indicated in *A–E*. Another aliquot of polysomes (*F*) was incubated at 0°C in buffer and EDTA (5 μmol/mg ribosomes) (14). L^P, large puromycin subunit; S^P, small puromycin subunit; L^E, large EDTA subunit; S^E, small EDTA subunit.

50-μl aliquots were layered on 5–20% sucrose gradients in 500 mM KCl, 5 mM $MgCl_2$, and buffer. Centrifugation: 2.6 hr at 20°C.

The data in Fig. 5 demonstrate the biological activity of the subunits isolated from hepatic ribosomes treated with puromycin. It can be seen that the small subunits had no activity; the large subunits alone showed some activity, but it amounted to less than 10% of the activity of the combined small- and large-subunit fractions. Polyphenylalanine synthesis was strictly poly(U)-dependent, i.e., there was no endogenous incorporation. It remains to be determined, however, how many of the recombined subunits participate in the polyphenylalanine synthesis, and we are now investigating this point with labeled puromycin. The number of [^{14}C]phenylalanine residues incorporated per pair of large and small subunits was estimated to be 5–10.

The apparently complete dissociation of polysomes by puromycin seen in Fig. 2*D* offers a simple and convenient method to obtain biologically active subunits. Complete dissociation into *biologically active* subunits is critically dependent on the complete release of nascent chains. If this is to be achieved by puromycin alone, a stringent set of conditions is required in order to assure a complete reaction of puromycin with the nascent chains. As was already pointed out, high ionic strength (Fig. 1) and, in particular, the ratio of monovalent ions to magnesium are important parameters; other factors to be considered include temperature, amount of magnesium bound to the ribosomes, and puromycin concentration. Since methods for the preparation of ribosomes vary widely, in particular with respect to the concentrations of monovalent ions and magnesium used, the results shown in Fig. 6 may not be directly applicable to ribosomes prepared by other methods or from other sources. In any case, experiments of the type shown in Fig. 6 will establish the conditions necessary to produce the complete dissociation of ribosomes isolated by different methods from other sources. In the experiments shown here (Fig. 6), the KCl concentration was kept constant at 500 mM during the incubation of polysomes with puromycin, whereas the magnesium concentration was varied from 10 to 0.5 mM. To facilitate comparison, we layered equal amounts of ribosomes on each sucrose gradient. It can be seen that dissociation of polysomes is incomplete at 10 mM magnesium (Fig. 6*A*). When the magnesium concentration is lowered to 2 mM (Fig. 6*C*) dissociation is complete. Further lowering of the magnesium concentration to 1 mM (Fig. 6*D*) does not change the situation significantly with respect to the subunits. Thus, at the given concentration of ribosomes (in our case, approximately 2 μmol of bound magnesium per mg), 500 mM KCl and a range from 1 to 2 mM magnesium during puromycin treatment cause optimal dissociation into subunits. If the magnesium concentration during the puromycin treatment is further lowered to 0.5 mM (Fig. 6*E*) a conversion of the large ribosomal subunit into a much slower-sedimenting peak, which sediments only slightly faster than the small subunit, is observed. The changes concomitant with this conversion or "derivativization" and also with the conversion of the small subunit caused by EDTA treatment of polysomes (Fig. 6*F*) will be reported elsewhere. It can be seen that the derivative of the large ribosomal subunit (Fig. 6*E*), which was found to be biologically inactive (data not shown here), sediments at the same rate as the biologically inactive large subunit obtained by EDTA treatment of polysomes (Fig. 6*F*).

These experiments established optimal conditions for complete dissociation by puromycin and revealed the importance of the ratio of monovalent ions to magnesium. The monovalent ion concentration of 500 mM, which we used, here, should be considered as somewhat arbitrary. Higher concentrations of monovalent ions would probably be as useful, when tested over a range of magnesium concentrations. In any case, the conditions described in Fig. 6*C* were successfully applied in preliminary experiments to ribosomes isolated from L cells and rabbit reticulocytes, and the conditions of Fig. 6*B* to ribosomes isolated from *E. coli*. In all cases, complete dissociation was achieved and biologically active ribosomal subunits were obtained.

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1. Martin, T. E., and I. G. Wool, *J. Mol. Biol.*, **42**, 151 (1969).
2. Lawford, G. R., *Biochem. Biophys. Res. Commun.*, **37**, 143 (1969).
3. Falvey, A. K., and T. Staehelin, *J. Mol. Biol.*, **53**, 1 (1970).
4. Petermann, M. L., A. Pavlovec, and J. B. Weinstein, *Fed. Proc.*, **28**, 725 (1969).
5. Dickman, S. R., and E. Bruenger, *Biochemistry*, **8**, 3295 (1969).
6. Blobel, G., and D. D. Sabatini, *J. Cell Biol.*, **45**, 130 (1970).
7. Blobel, G., and V. R. Potter, *J. Mol. Biol.*, **26**, 293 (1967).
8. Eagle, H., *Science*, **130**, 432 (1959).
9. Blobel, G., and V. R. Potter, *Biochim. Biophys. Acta*, **166**, 48 (1968).
10. Hultin, T., *Biochim. Biophys. Acta*, **123**, 561 (1966).
11. Maden, B. E. H., and R. E. Monro, *Eur. J. Biochem.*, **6**, 309 (1968).
12. Lucas-Lenard, J., and A. L. Haenni, *Proc. Nat. Acad. Sci. USA*, **63**, 93 (1969).
13. Williamson, A. R., and R. Schweet, *J. Mol. Biol.*, **11**, 358 (1965).
14. Tashiro, Y., and P. Siekevitz, *J. Mol. Biol.*, **11**, 149 (1965).