*THE FORMATION AND STABILIZATION OF 30S AND 50S RIBOSOME COUPLES IN ESCHERICHIA COLI**

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It has been widely accepted that 30*S* and 50*S* ribosomes, as soon as they are formed, can join together to form a 70*S* "monomer."[1] Polypeptide chains would then be formed on each monomer as it moves across each of a succession of mRNA molecules.[2] However, recently we found that rapidly lysed cells can yield extracts containing 30*S* and 50*S* ribosomes and polyribosomes, but few, if any, monomers.[3] It was then shown that newly labeled ribosomal RNA arrives in free 30*S* and 50*S* ribosomes and those in polyribosomes at the same rate,[4] implying that the free particles and those in polyribosomes are in rapid exchange. We therefore suggested[3, 4] that all ribosomal particles, including the newly formed ones, cycle periodically through a free pool of 30*S* and 50*S* ribosomal particles. These would join together in 30*S*–50*S* couples as they add on to mRNA, and come apart when translation of the mRNA is complete. Thus, no stable pool of 70*S* monomers would exist in growing cells.

This suggested cycle of ribosomal particles *in vivo* maintained that all 30*S* and all 50*S* ribosomes are equivalent. However, in extracts, the free 30*S* and 50*S* ribosomes seemed to be different from those in polyribosomes: in 0.01 *M* Mg^{2+}, the free ribosomes formed no 70*S* monomers,[3, 5] while the ribosomes derived from polyribosomes or 70*S* monomers by prior dissociation in low Mg^{2+} ("derived ribosomes") could associate to form monomers.[3, 5]

Here we report results suggesting that derived and free ribosomes *are intrinsically equivalent*, but that derived particles are additionally stabilized in 70*S* form by the peptidyl tRNA that they bear; and while 0.01 *M* Mg^{2+} is insufficient to promote coupling of free 30*S* and 50*S* particles, they form 70*S* monomers in conditions required for the initiation of protein syntheis. From these results, a more detailed cycle of ribosome function is constructed.

Materials and Methods.—5-H^3-uracil (22 mc/μmole), 2-C^{14}-uracil (58 mc/mmole), and C^{14}-L-phenylalanine (330 mc/mmole) were purchased from Schwarz BioResearch, Inc. Puromycin dihydrochloride was obtained from Nutritional Biochemicals Corporation. Poly-U and poly-AUG (1:1:1) were the Miles Laboratory products.

Crude extracts (S-30) and soluble enzymes (S-100) were prepared from *E. coli* K12 3000 grown in broth medium, harvested with ice, ground with alumina, and extracted in 3 ml/gm cells of 10 mM tris-HCl, pH 7.5, 10 mM magnesium acetate, 6 mM β-mercaptoethanol.[6] The extracts were treated with 1 μg/ml DNase (Worthington Biochemical Co.). 30*S*, 50*S*, and 70*S* ribosomes were prepared by zonal centrifugation of 1.0 ml of S-30 in 15–30 per cent sucrose gradients containing 10 mM tris, pH 7.5, 10 mM magnesium acetate. Gradients were either 28 ml, centrifuged at 5°C for 14 hours at 87,000 $\times$ *g* in a Beckman model L-2 SW25.1 rotor, or 13 ml, centrifuged at 5°C for 5 hours at 260,000 $\times$ *g* in an International model B-60 SW265 rotor. 30*S*, 50*S*, and 70*S* peak fractions were pooled and di-

alyzed against tris-Mg^{2+} buffer for two hours at 4°C before use. To aid in identifying ribosomes in sucrose gradients, they were purified from extracts of cells uniformly labeled with H^3-uracil (100 μc/gm cells), or C^{14}-uracil (20 μc/gm cells). tRNA was purified by the method of Tissières.[7]

Polyphenylalanine synthesis directed by poly-U was carried out in reaction mixtures containing, per ml: 10 μmoles tris-HCl, pH 7.5; 100 μmoles $(NH_4)_2SO_4$; 1μ mole adenosine 5′-triphosphate (ATP); 0.5 μmole guanosine 5′-triphosphate (GTP); 6 μmoles phosphoenolpyruvate; 40 μg pyruvate kinase; 2 μc C^{14}-phenylalanine; 40 μg poly-U; and 0.2 ml S-100. Except where otherwise noted, 16 μmoles magnesium acetate was also added. 70*S* (120 μg), 50*S* (80 μg), and 30*S* (40 μg) ribosomes were added as indicated for each experiment. The reaction was carried out at 30°C and terminated as indicated in the text, either by chilling in an ice bath or by the addition of 4 ml of 5 per cent trichloracetic acid to portions, usually 50 μl, of the reaction mixture. Incorporated C^{14}-phenylalanine was measured in a Packard Tri-carb spectrometer on acid-precipitated samples after boiling 20 minutes at 90°C. When radioactivity in H^3-RNA and C^{14}-polyphenylalanine was to be determined in the same fraction, one portion was plated and washed in ice-cold acid before counting. To recheck the cpm in polyphenylalanine, a second portion was plated after boiling in acid.

Results.—Puromycin frees ribosomal particles from monomers: In preliminary experiments, the free and derived 30*S* and 50*S* particles were respectively labeled with H^3- and C^{14}-uracil, and their relative capacity to join in 70*S* couples was tested. Free and derived 30*S* ribosomes entered the 70*S* form equally well, but only the derived 50*S* ribosomes could join with 30*S*, and did so selectively even in the presence of a 100-fold excess of free 50*S*. The derived 50*S* particles therefore appeared to differ from free 50*S* ribosomes. The difference could be peptidyl tRNA, for when shear and nuclease action generate 70*S* monomers from polyribosomes, tRNA and soluble enzymes are easily washed off, but each monomer seems to retain a peptidyl tRNA, bound tightly to the 50*S* particle[8] and, relatively weakly, to the 30*S* ribosome.[9, 10] Peptidyl tRNA might act as a clasp to help bind 30*S* and 50*S* particles together and could promote the reformation of 70*S* monomers from dissociated 50*S* and 30*S* particles.

Puromycin acts on isolated "70*S* monomers" or 50*S* that bear peptidyl tRNA and releases a soluble fraction of the peptidyl chains as a puromycin derivative.[8, 11–14] Therefore, if peptidyl tRNA helps to stabilize a 30*S*–50*S* couple, puromycin treatment could lead to their dissociation.

Figures 1 and 2 show the results of such experiments, analyzed by zonal sedimentation in sucrose gradients. 70*S* monomers treated with puromycin dissociated into 30*S* and 50*S* particles, even in 0.01 *M* Mg^{2+} (Figs. 1*a* and *b*). In the experiments partially depicted in Figures 1 and 2, in which about 83 per cent and 64 per cent of the polyphenylalanine was released by puromycin treatment in high Mg^{2+}, about 80 per cent and 72 per cent of the 70*S* ribosomes were dissociated to 30*S* and 50*S* particles. Five other such experiments were carried out, and the release of about 53, 54, 63, 70, and 88 per cent of the polyphenylalanine was accompanied by dissociation of about 45, 62, 56, 67, and 79 per cent of the 70*S* monomers. Thus we have confirmed the observation that the efficiency of formation and release of peptidyl varies from 50 to 90 per cent in different experiments.[11] Most important,

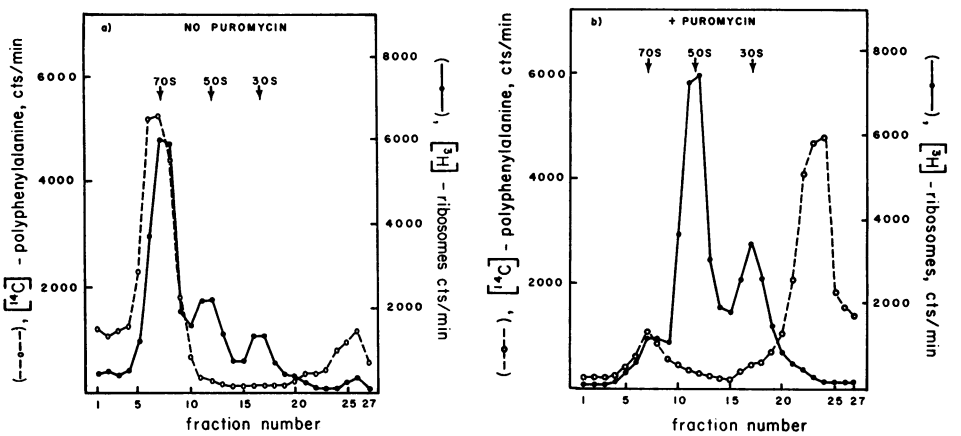

FIG. 1.—Release of 30*S* and 50*S* ribosomes from 70*S* monomers by treatment with puromycin. Synthesis of polyphenylalanine was carried out on 120 μg H^3-labeled 70*S* ribosomes in 1 ml (as in *Materials and Methods*, except with 86 mM KCl instead of ammonium sulfate). After the 15-min incubation, the sample was chilled in an ice bath. To 0.5 ml, 500 μg puromycin, neutralized with tris, was added. The sample treated and that untreated with puromycin were left on ice for 30 min. Puromycin treatment was carried out at 0°C to minimize the reinitiation of polypeptide chains that can occur at higher temperatures and reform peptidyl tRNA.[29] After dialysis for 4 hr at 4°C, with one change of buffer, against 2 liters of 10 mM tris, pH 7.5, 10 mM magnesium acetate, the samples were analyzed in 13-ml sucrose gradients (see *Materials and Methods*). (—●—), H^3-uracil; (- -O- -), C^{14}-polyphenylalanine.

the correlation between the percentage of polyphenylalanine released and the percentage of ribosomes dissociated suggests that every time a peptidyl chain is released, a 70*S* monomer dissociates.

When particles are all dissociated to 30*S* and 50*S* ribosomes in 0.1 mM Mg^{2+}, and then returned to 0.01 *M* Mg^{2+}, those bearing peptidyl tRNA, in samples treated or untreated with puromycin, tend to reassociate selectively to the 70*S* form. In Figure 2, the action of puromycin has not been complete, and derived particles bearing peptidyl tRNA can be obtained by dialysis in low Mg^{2+} from the monomers remaining after puromycin treatment (Fig. 2*c*). When the dialyzed sample treated with puromycin and the untreated control are brought back to high Mg^{2+}, the monomers in the control all reform (Figs. 2*a* and *b*), and the peptidyl tRNA that survives puromycin treatment and remains bound to the 50*S* ribosomes in low Mg^{2+} (Fig. 2*c*) moves back into the 70*S* form in high Mg^{2+} (Fig. 2*d*). The particles that remain free, even in 0.01 *M* Mg^{2+}, after puromycin treatment tend to be those that have lost the peptidyl tRNA. Formally speaking, therefore, "free" 30*S* and 50*S* particles have been made from derived ones.

How can free ribosomal particles function? While 30*S* and 50*S* couples can be stabilized by peptidyl tRNA, couple formation during initiation must *precede* the synthesis of peptidyl tRNA. Conditions were therefore sought that induce free particles to come together and participate in polypeptide synthesis.

In the usual assay conditions for polyphenylalanine formation, purified free particles did not form 30*S* and 50*S* couples and functioned very little and variably. However, they function well in the presence of higher levels of Mg^{2+} (Fig. 3). The Mg^{2+} probably helps to form the initiating complex (ref. 15 and below).

With the knowledge that high Mg^{2+} and the usual factors required for protein

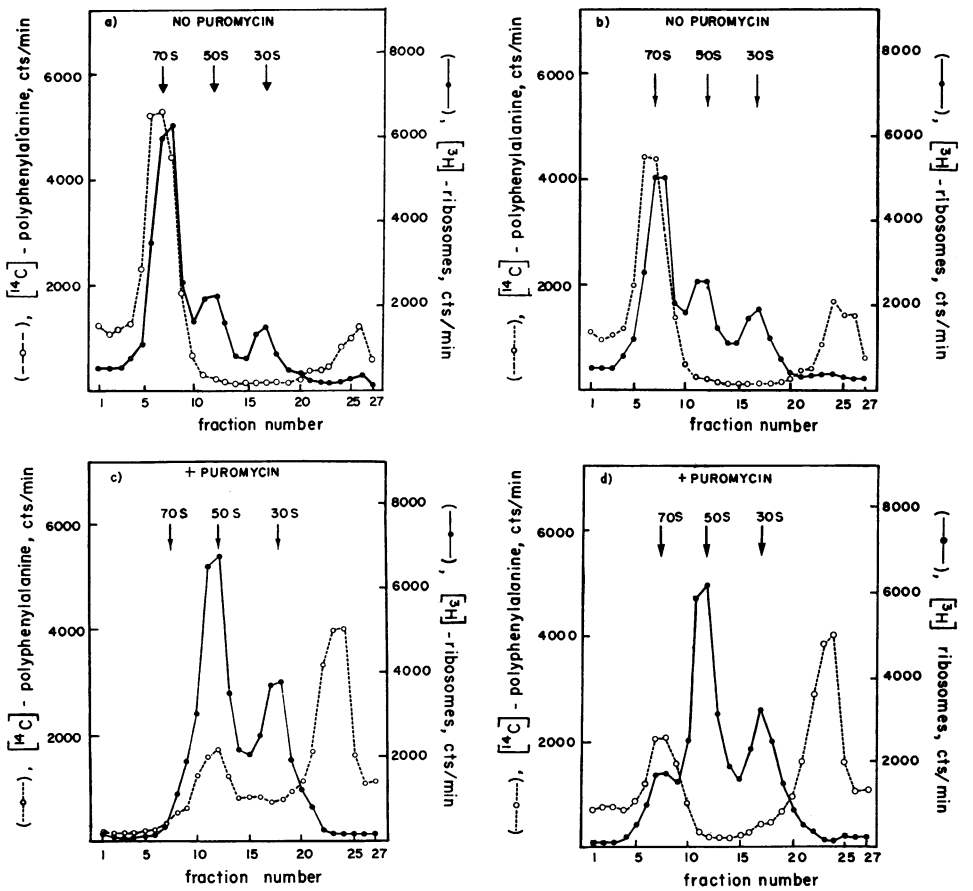

FIG. 2.—Selective reassociation of ribosomal particles bearing peptidyl tRNA. The experimental design as in Fig. 1, except that after incorporation of C^{14}-phenylalanine into protein, replicate samples treated or untreated with puromycin were dialyzed against 0.1 mM Mg^{2+} (low Mg^{2+}) to dissociate all remaining 70*S* particles into 30*S* and 50*S* ribosomes. Portions of each dialyzed sample were then brought back to 10 mM Mg^{2+} (high Mg^{2+}) to reassociate ribosomes that could come back together, and the samples analyzed in sucrose gradients. (*a*) Sample not treated with puromycin, undialyzed; (*b*) sample not treated with puromycin, dialyzed in low Mg^{2+} and returned to high Mg^{2+}; (*c*) sample treated with puromycin, dialyzed in low Mg^{2+}, and analyzed in a sucrose gradient containing low Mg^{2+} to display all ribosomes in 30*S* and 50*S* form; (*d*) sample treated with puromycin, dialyzed against low Mg^{2+}, and restored to high Mg^{2+}, showing formation of a small amount of 70*S* particles which is in proportion to the 50*S* particles that retained polypeptide after puromycin (cf. panel *c*).

synthesis in extracts are *sufficient* to activate free ribosomal particles, the system was then dissected to find the necessary conditions for couple formation.

Formation of 30*S*–50*S* couples from one free H^3-labeled 30*S* and one free C^{14}-labeled 50*S* was observed in gradient analyses, some of which are shown in Figure 4. The absolute requirements for couple formation include mRNA, tRNA, K^+, and Mg^{2+}. The last two deserve special note. K^+ is always required for polypeptide formation, and cannot be replaced by Na^+;[16] here only K^+ permits couple formation.

The level of Mg^{2+} required for couple formation depends on the mRNA used:

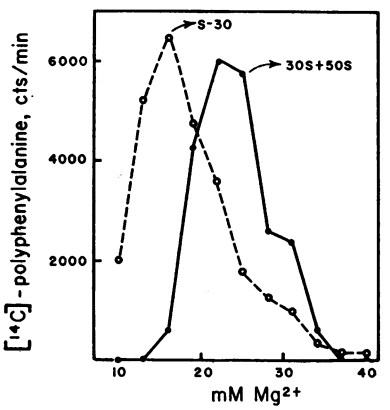

FIG. 3.—Response to Mg^{2+} of polyphenylalanine synthesis in a crude extract (S-30), or on purified free 30*S* and 50*S* ribosomes. Polyphenylalanine formation was tested in 50-μl reaction mixtures at the Mg^{2+} concentrations indicated, with 2 μg free 30*S* and 4 μg free 50*S* ribosomes (—●—), or S-30 containing 6 μg ribosomes (- -○- -).

0.02 *M* Mg^{2+} with poly-U as mRNA, 0.01 *M* Mg^{2+} when poly-AUG is the mRNA. The Mg^{2+} requirement probably is determined by the necessity for mRNA-directed binding of tRNA to the ribosome (see *Discussion*). For example, efficient binding of phenylalanyl tRNA directed by poly-U requires 0.02 *M* Mg^{2+} (ref. 17); here, 0.02 *M* Mg^{2+} is also required for couple formation, and 0.01 *M* is not enough (Figs. 4*a* and *c*). In an analogous way, when the natural initiating triplet AUG[18–20] directs the binding of formylmethionyl tRNA,[21] 0.01 *M* Mg^{2+} is adequate; here, with poly-AUG as mRNA, 0.01 *M* Mg^{2+} is enough to support couple formation (Fig. 4*a*). As Nirenberg and Leder have shown,[17] the binding of tRNA can be slowed or blocked if the concentrations of Mg^{2+}, tRNA, or mRNA are lower than optimal, or if the binding reaction is carried out at 0°C instead of 24° to 37°C. One or more of these possibilities can explain why couples did not reform after puromycin treatment in the conditions of Figures 1 and 2.

Although tRNA was obligate for couple formation, it was found in subsequent experiments that stripped tRNA functioned as well as charged. In the cell an uncharged tRNA in such a complex would presumably be displaced by a charged molecule, or alternatively might be aminoacylated on the ribosome in order for peptide bond formation to begin. If any other factors, like ATP, GTP, or initiating factors,[22–24] are required for the complex formation, they must have been present in the preparations used.

Discussion.—70*S* monomers that persist, moving from one mRNA to another, no longer seem an obligate feature of protein synthesis in growing bacteria. The 30*S*–50*S* couples in a polyribosome do seem to be stabilized in a 70*S* form; but at least in part, the tRNA bearing the nascent polypeptide chain seems to be the stabilizing agent, for when the peptidyl tRNA is removed (Fig. 1), the 30*S*–50*S* partners tend to dissociate. We suggest that the couples themselves are intrinsically unstable, and that in the cell, when the peptidyl tRNA is cleaved to release a finished polypeptide chain, the couple is destabilized. Such a notion is consistent with our earlier studies[3] of cells harvested in absence of chloramphenicol to permit a small amount of protein synthesis to continue. When pulse-labeled radioactive proteins were completed, their release from polyribosomes was accompanied by the dissociation of 30*S*–50*S* complexes.

According to the proposed model, some of the time, and likely every time trans-

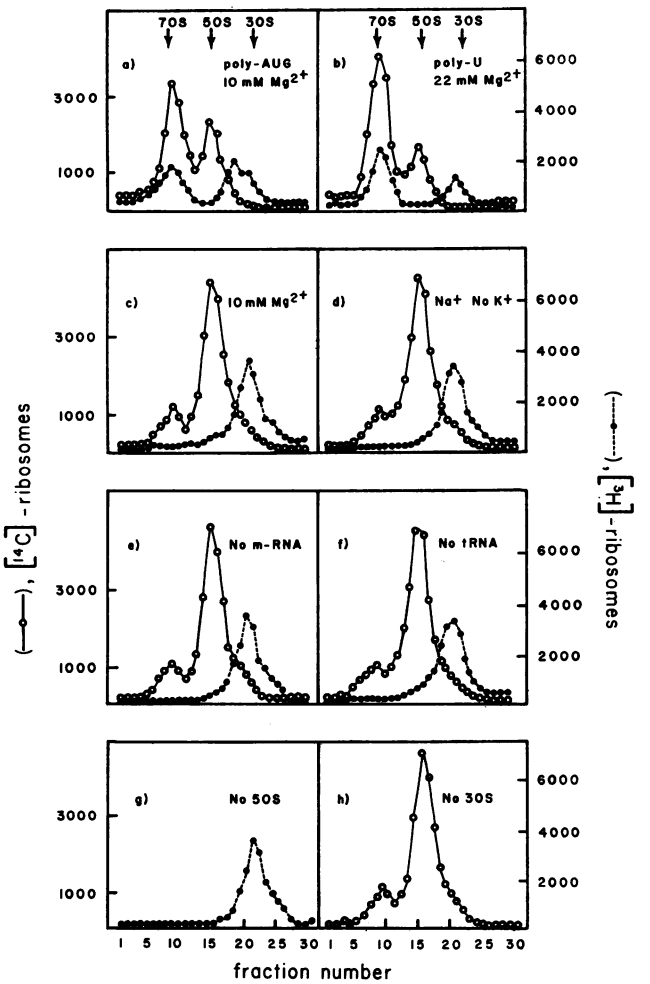

FIG. 4.—30*S*–50*S* couple formation directed by poly-U and poly-AUG. In each of a series of 0.5-ml samples, 20 μg purified H^3-labeled free 30*S* (- -●- -) and 40 μg C^{14}-labeled free 50*S* ribosomes (—O—) were incubated for 15 min at 30°C, and the formation of 70*S* monomers gauged by zonal sedimentation analysis in 13-ml sucrose gradients. Incubation mixtures that contained only 30*S* (*g*) or only 50*s* (*h*) subunits showed only small amounts of contaminating 70*S* monomers. Panels (*a*) and (*b*) show analyses of mixtures that contained all the necessary requirements for formation of an appreciable fraction of the 30*S*–50*S* couples, including: 10 mM tris, pH 7.5; 86 mM KCl; 125 μg tRNA; mRNA and Mg^{2+}. In (*a*) the mRNA was 150 μg of poly-AUG and 10 mM Mg^{2+} was adequate; in (*b*) the mRNA was 75 μg of poly-U and 22 mM Mg^{2+} was used. In panels (*c*), (*d*), (*e*), and (*f*), control samples with poly-U but lacking one or another of the requirements showed no measurable couple formation. In panel (*d*), 86 mM NaCl was added in place of KCl.

lation of an mRNA begins, an "initiation complex" has to form, involving the coming together of a free 30*S* and a free 50*S* particle. Our results show that such a complex forms under appropriate conditions, without difficulty (Fig. 4).

Suzuka, Kaji, and Kaji,[25] as well as Pestka and Nirenberg,[26] had already shown that a purified 30*S* subunit, in the presence of mRNA, can bind one aminoacyl tRNA. Other results of the two groups are somewhat at variance, but it seems

clear that in the presence of K^+ and 50*S* particles a second tRNA can bind. Such results, combined with ours (refs. 3 and 4, and Fig. 4), suggest that initiation can occur on free 30*S* and 50*S* ribosomes either with formylmethionyl tRNA (F-met tRNA) in 0.01 *M* Mg^{2+} or with other aminoacyl tRNA's in higher Mg^{2+} (about 0.02 *M*). In the *E. coli* cell, where F-met tRNA is probably the first tRNA to bind,[18–20] the sequence of reactions would be as follows:

(1) A 30*S* subunit attaches to mRNA and

(2) F-met tRNA is bound opposite the initiating AUG codon.

(3) In the presence of K^+, the 50*S* ribosome and a second tRNA, directed by the codon next to AUG,[20] adds to the complex.

(4) The first peptide bond is formed.

The proposed model provides a more detailed mechanism for the rapid exchange of particles in and out of polyribosomes observed in our earlier studies with whole cells.[4] Other experiments that support such a model have recently been performed by Kaempfer and Meselson,[27] and by Nomura and Lowry.[28] The first authors have demonstrated conclusively that during exponential growth of *E. coli* the ribosomal particles exchange partners. This was done by transferring cells labeled with heavy isotopes into medium containing light isotopes, and finding 70*S* monomers that contained heavy 50*S* and light 30*S* or vice versa. Nomura and Lowry found that phage RNA can direct the binding of F-met tRNA only on 30*S* ribosomes, not on 50*S* or 70*S* particles, strongly suggesting that initiation can only take place on free 30*S* particles.

It should be emphasized that, according to this model, in extracts of exponentially growing *E. coli* cells, there should be at least three types of monomers: type 1, those just formed on mRNA during initiation of protein synthesis, but not yet bearing peptidyl tRNA [analogous to those formed from free 30*S* and 50*S* particles (see Fig. 4)]; type 2, those 30*S*–50*S* couples in polyribosomes or the same couples released by degradative action, bearing a nascent polypeptide chain; and type 3, those that have completed a protein chain and are ready to leave the messenger RNA and dissociate. It is not yet clear what conditions may determine the residual stability of each class in extracts. While we have found peptidyl tRNA to be a critical factor in holding together the functioning 70*S* form (type 2), other factors, such as "initiating factors,"[22–24] might be involved in forming type 1, or in stabilizing types 2 and 3. The precise distribution of ribosomes among polyribosomes, 70*S* monomers, and free subunits in lysates would then depend on the balance of various initiation, completion, and degradative processes during harvest and lysis of cells.

Summary.—Extracts of *Escherichia coli* contain free 30*S* and 50*S* ribosomal particles. These do not spontaneously associate, but can couple to form 70*S* monomers in presence of mRNA, tRNA, and K^+ and Mg^{2+} ions (about 0.022 *M* Mg^{2+} when poly-U is the mRNA used, and 0.01 *M* Mg^{2+} when poly-AUG is the mRNA). Incorporation of phenylalanine directed by poly-U on free 30*S* and 50*S* particles, like couple formation, requires about 0.02 *M* Mg^{2+}. Once protein synthesis has begun, the peptidyl tRNA seems to help stabilize the 30*S*–50*S* couple, for when the peptidyl chain is released as the puromycin derivative, the 30*S* and 50*S* particles separate.

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