Studies on Native Ribosomal Subunits from Rat Liver. Purification and Characterization of a Ribosome Dissociation Factor†

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ABSTRACT: A population of free, native ribosomal 40S subunits, that do not react with 60S subunits to form 80S ribosomes, has been identified in the postmicrosomal fraction of rat liver homogenates. A protein (IF-3) has been purified from high salt (0.88 M KCl) extracts of native 40S subunits by gradient centrifugation and by ammonium sulfate fractionation; it prevents the reassociation of subunits and to a limited extent dissociates ribosomes to subunits. The activity is measured by ultracentrifugation of the reaction products on linear sucrose gradients, or with an assay developed in this laboratory that couples dissociation with the 60S-specific peptidyltransferase reaction; the latter procedure measures the amount of 60S subunits released from ribosomes or remaining in incubations in the presence of IF-3. Dissociation factor activity is recovered from most of the particles that are resolved by zonal centrifugation of the total "native subunits" obtained from the postmicrosomal fraction; the highest concentration of IF-3, however, appears to be associated with native 40S subunits. The purified dissociation factor IF-3 is composed of about ten polypeptides and the molecular weight is estimated to be between 500 000 and 700 000, on the basis of glycerol and cesium chloride gradient centrifugation. When purified 40S subunits react with IF-3 or when 80S ribosomes are dissociated by IF-3, a product is formed which is dependent on the concentration of the protein factor and has the characteristics of a 40S·IF-3 complex; centrifugation of the complex on sucrose and cesium chloride gradients suggests that the complex consists of 1 equiv of each of the two components. Although dissociation factor IF-3 appears to react in a specific manner with free or ribosome-associated 40S subunits, the reaction with subunits differs in several respects from that with ribosomes. The dissociation factor also appears to interact with 60S subunits but multiple complexes are formed, some with more than 1 IF-3 equiv per 60S particle. The IF-3 converts 40S dimers (55S particles) to the 40S·IF-3 complex and dissociates free, native 80S particles present in the postmicrosomal fraction, but it does not affect polysome-associated ribosomes engaged in protein synthesis.

Ribosomes exist in the cell in the form of polysomes, monomeric ribosomes, and free or "native" subunits. The occurrence of a ribosome cycle in which subunits participate in one of the stages in protein synthesis has been postulated, and evidence for such a cycle in mammalian cells has been presented (Kaempfer and Meselson, 1969; Adamson et al., 1969; Baglioni et al., 1969). The ribosome cycle has been reviewed recently by Kaempfer (1974) and by Davis (1974). After termination of the polypeptide chain, ribosomes leave the polysome complex as monomeric units (Davis, 1971) or as subunits (Kaempfer and Kaufman, 1972; Ayuso-Parilla et al., 1973). Since initiation of protein synthesis proceeds through the interaction of the small ribosomal subunits with the initiation components, small subunits must be available at some stage of the ribosome cycle. At near-physiological concentrations of magnesium ions, however, the stability of naturally occurring monomers is high, as is the affinity of polysome-derived subunits for each other. Evidence for ribosome dissociation in bacteria and yeast has been reported by Kaempfer et al. (1968) and Kaempfer and Meselson (1969). A ribosome dissociation factor has been found in the high salt "wash" of *E. coli* ribosomes, which is suggested to function in vivo to provide subunits for the process of initiation (Subramanian et al., 1968, 1969); this dissociation activity is expressed by purified IF-3 from prokaryotes (Sabol and Ochoa, 1971). Ribosome dissociation activity has also been reported in the ribosomal "wash" fraction or partially purified preparations of yeast (Petre, 1970), reticulocytes (Lubsen and Davis, 1972; Merrick et al., 1973; Mizuno and Rabinowitz, 1973; Favelukes et al., 1973; Chen et al., 1974), and ascites cells (Nakaya et al., 1973), and in extracts obtained from native subunits of Ehrlich ascites cells (Ayuso-Parilla et al., 1973), reticulocytes (Lubsen and Davis, 1974), a rat liver fraction rich in native subunits (Lawford et al., 1971), and rat liver native 40S subunits (Sadnik et al., 1975).

Native 40S subunits isolated by zonal ultracentrifugation from the postmicrosomal supernatant fraction of rat liver differ in several respects from subunits derived from polysomes by dissociation in high salt. Whereas polysome-derived 40S subunits react quantitatively with 60S subunits in the absence of nonribosomal protein factors, a significant portion of the native 40S subunits does not interact directly with 60S subunits to form 80S ribosomes (Sadnik et al., 1975). Preliminary experiments in this laboratory have also shown that incubation of 80S ribosomes with a high salt extract prepared from native 40S subunits leads to the release of ribosomal subunits. These data suggest that a ribosome dissociation factor is present on some native 40S subunits. This communication describes the purification and some of the structural and biological characteristics of a high-molecular-weight complex from rat liver native ribosomal subunits, that prevents the reassociation of derived 40S and 60S subunits and dissociates 80S ribosomes. This "dissociation" factor appears to be similar to the large multiprotein complex required for initiation of globin synthesis in vitro (Schreier and Staehelin, 1973a–c; Prichard and Anderson, 1974; Staehelin et al., 1975; Sundkvist and Staehelin,

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1975; Freienstein and Blobel, 1975; Safer et al., 1976; Benne and Hershey, 1976).

Experimental Procedures

Preparation of Derived and Native Ribosomal Subunits and High-Salt Extracts of Native Subunits. Derived ribosomal subunits (d40S and d60S) were prepared from purified ribosomes by dissociation in 0.88 M KCl solutions (Martin and Wool, 1968; Gasior and Moldave, 1972; Sadnik et al., 1975), and resolved by zonal ultracentrifugation (Sadnik et al., 1975). Free, native subunits were sedimented from the postmicrosomal supernatant fraction ("total native subunits") and resolved by zonal centrifugation as described (Sadnik et al., 1975). The free 40S subunits ("n40S subunits") were less than 10% contaminated with 60S subunits; however, because of the extensive and variable amounts of ferritin, it was difficult to obtain an exact value for the concentration of pure 40S ribonucleoprotein in these preparations.

Resolved n40S subunits were extracted with solutions containing 0.88 M KCl and 12.5 mM $MgCl_2$ essentially as described (Sadnik et al., 1975). The extract was vacuum dialyzed at 2 °C for 10 h against a solution containing 10 mM Tris[1]-HCl (pH 7.2), 50 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM EDTA, centrifuged at 1500*g* for 10 min to remove any insoluble material, and the supernatant ("n40S extract") containing dissociation factor IF-3 was stored frozen at −60 °C in aliquots.

An extract was similarly prepared from the total native subunit fraction. This preparation was composed of 60% n40S subunits and 40% n60S plus n80S particles. The total native subunits were partially purified by centrifugation through a discontinuous (0.5–1.0 M) sucrose gradient which removed about 50% of the nonribosomal proteins, including most of the ferritin.

Incubation Conditions and Analyses by Sucrose Gradient or Cesium Chloride Centrifugation. Approximately 16 pmol of d40S, n40S, or d60S subunits (0.3, 0.6, and 0.5 A_{260} unit, respectively) were incubated individually or together, with or without n40S extract or purified dissociation factor IF-3, in a solution containing 30 mM morpholinopropanesulfonate-KOH (Mops, pH 7.2), 4.0 or 5.6 mM $MgCl_2$, 60 mM NH_4Cl (or KCl), and 2 mM dithiothreitol. Incubations were at 37 °C in a total volume of 0.1 or 0.5 mL; similar results were obtained when 0.1 and 0.5 mL volumes were used. In some experiments, the d40S and d60S subunits were allowed to react for varying periods of time at 37 °C, prior to the addition of dissociation factor; this procedure was used to measure ribosome dissociation. In other experiments d40S and IF-3 were incubated at 37 °C, prior to the addition of d60S subunits; this procedure was used to measure subunit reassociation.

When the reaction mixtures were analyzed by sucrose gradient centrifugation, they were layered on 12 mL of linear (10–30%) sucrose gradients, centrifuged at 200 000*g* for 3.5 h or 50 000*g* for 14 h (SW 41 rotor, Spinco), and analyzed as described previously (Sadnik et al., 1975). The optical density data are presented in the figures in the form of spectrophotometric scans (at 254 nm) obtained from the continuous recording apparatus. In all the figures, sedimentation is toward the right. The concentrations of ribosomes and subunits in the gradient were estimated from the areas under the peaks in the optical density patterns.

When the reaction mixtures were analyzed by CsCl centrifugation, the procedures described by Hirsch et al. (1973) were used with minor modifications. The samples were "fixed" with 3.2–3.4% formaldehyde, layered over 5 mL of a buffered salts–CsCl solution (density 1.60), and centrifuged at 115 000*g* (SW 41 rotor, Spinco) for 24 h. After centrifugation, the contents of the tubes were scanned spectrophotometrically at 254 nm and fractionated and the refractive indices of individual fractions measured. Calculation of the protein–RNA composition of the particles obtained from the gradient, based on buoyant density values, was carried out according to the formula of Perry and Kelley (1966).

Incubation Conditions and Analysis by the Peptidyltransferase Assay. A multiple-incubation procedure was used to determine the concentration of 60S subunits released in incubations containing ribosomes or remaining in incubations containing subunits, in the presence of dissociation factor. The reaction with IF-3, causing ribosome dissociation or preventing reassociation of subunits, was coupled to the 60S-specific transpeptidation reaction between acylaminoacyl-tRNA and puromycin. Under the conditions employed, 60S subunits, but not 40S subunits or 80S ribosomes, formed acetyl[^{3}H]methionylpuromycin. The procedure has been described in detail (Thompson et al., 1976). The first incubation contained d40S, d60S, or preformed 80S particles, with or without n40S extract or purified IF-3. Incubations were for varying periods of time at 37 °C in a volume of 0.05 mL (or 0.2 mL if glycerol was present from glycerol gradients used for purification). To the first incubation were added acetyl[^{3}H]methionyl-$tRNA_m$ and a salt–ethanol–puromycin solution to obtain the following concentrations: 0.3 M KCl, 0.8 mM puromycin, 33% ethanol, 4 mM $MgCl_2$, and 1.4 mM dithiothreitol. The acetyl-[^{3}H]methionyl-$tRNA_m$ was prepared by acetylation of isotopically labeled methionyl-tRNA with acetic anhydride; the specific radioactivity of the acetyl[^{3}H]Met-$tRNA_m$ was 6700 cpm/μg of aminoacyl-tRNA and 3450 cpm/pmol of esterified methionine. The solutions, in a total volume of 0.15 mL (or 0.6 mL for those samples containing glycerol), were incubated at 20 °C for 10 min. At the end of the second incubation, the reactions were analyzed for acetyl[^{3}H]methionylpuromycin (Leder and Bursztyn, 1966; Maden and Monro, 1968; Thompson and Moldave, 1974; Thompson et al., 1976). The results are expressed as picomoles of labeled methionine-containing product, after subtraction of the values obtained in control incubations without puromycin.

Results

A brief incubation of derived 40S and 60S ribosomal subunits led to the quantitative formation of 80S ribosomes as detected by sucrose gradient centrifugation (Figure 1A). When native 40S subunits, present in the free form in the postmicrosomal fraction of rat liver, were incubated with d60S subunits, only a portion of them combined to form ribosomes (Figure 1B). Some 80S particles were obtained in the gradient, but approximately half of the n40S population did not combine with the large subunit, although 60S particles were present in excess as evidenced by the presence of material in the 60S monomer and the 90S dimer peaks in the gradient. The sedimentation pattern of n40S subunits alone was presented in a previous communication (Sadnik et al., 1975). Although not shown here, the amount of 40S subunits that reacted with 60S subunits to form ribosomes increased significantly when the n40S subunits were extracted with high KCl solutions. A gradient pattern similar to that shown in Figure 1B was obtained when a limiting amount of n40S extract was incubated

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; poly(U),poly(uridylic acid); Mops, 3-(*N*-morpholino)propanesulfonic acid.

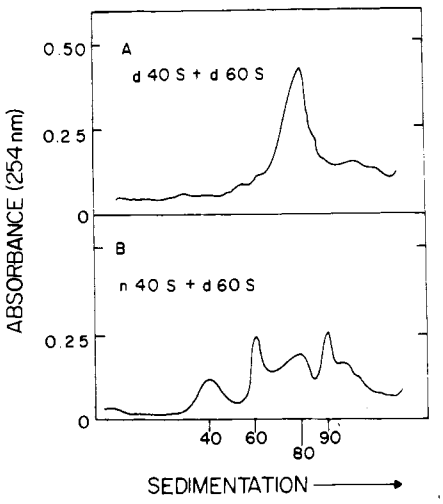

FIGURE 1: Sedimentation patterns of derived 60S subunits plus derived 40S (A) or native 40S (B) subunits on linear (10–30%) sucrose gradients, as described in the text. Incubations containing 16 pmol of d60S subunits plus 16 pmol of d40S or n40S subunits were for 30 min at 37 °C prior to gradient centrifugation. As noted in the horizontal axis, the ultraviolet-absorbing material in A sedimented in the region corresponding to 80 S. The main peaks detected in B had values corresponding to about 40, 60, 80, and 90 S, in that order, in the direction of sedimentation.

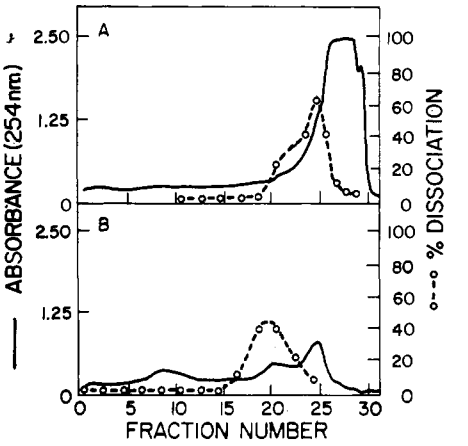

FIGURE 2: Gradient purification of IF-3. (A) Approximately 0.5 mL of n40S extract containing 9 mg of protein was layered on and centrifuged through 10 mL of linear 10–30% glycerol gradients (over 2 mL of a 60% glycerol cushion), containing the following components: 10 mM Tris-HCl buffer (pH 7.6), 2 mM $MgCl_2$, 50 mM KCl, and 1 mM dithiothreitol; centrifugation at 200 000*g* was for 16 h at 2 °C. Analyses for absorbance (solid line) and for dissociation factor activity (open circles) were as described in the text; the IF-3 activity was determined in 0.1-mL samples from each fraction, by sucrose gradient centrifugation; some fractions were spot-checked with the coupled peptidyltransferase procedure. (B) The fractions from several gradients, containing IF-3 activity, were pooled, concentrated by vacuum dialysis, and 4.5 mg of protein was centrifuged through glycerol gradients essentially as described above, except that the KCl concentration was raised to 0.5 M and the time of centrifugation was 24 h. Sedimentation in both gradients was toward the right. Most of the protein that sedimented faster than IF-3, fractions numbered 25 and greater, appeared to be ferritin.

with preformed 80S ribosomes (Sadnik et al., 1975); small ribosomal subunits as well as monomers and dimers of the large subunit were obtained from the ribosomes. These results and those reported previously indicated that n40S subunits contained an activity (IF-3), extracted with high-salt solutions, that prevented the interaction between subunits and caused dissociation of ribosomes.

A number of activities, in addition to the dissociation factor, were also detected in the n40S extract; evidence has been obtained (Sadnik et al., 1975) for the presence of a specific Met-$tRNA_f$ deacylase and for an activity (IF-1) that stimulated the template-dependent, GTP-independent binding of initiator-tRNA to d40S subunits. Purification and characterization of these protein factors and of an additional one (IF-2) in the n40S extract which interacted with Met-$tRNA_f$ in the presence of GTP but did not require template for reaction with 40S subunits, as described by others (see review by Weissbach and Ochoa, 1976), will be reported subsequently.

Resolution of the dissociation factor from the other activities and purification was carried out by gradient centrifugation. When the n40S extract was centrifuged through 10–30% linear glycerol gradients containing 50 mM KCl (over a cushion of 60% glycerol), for 16 h at 200 000*g* (Figure 2A), the bulk of the protein and the dissociation factor activity were recovered toward the bottom of the gradient, in fractions numbered 21–27. Other activities, such as the deacylase and IF-1, as well as a number of other proteins constituting only a small percent of the total protein in the extract, were recovered in the top half of the gradient. Under these conditions, only about 25% of the IF-3 activity (as determined by the sucrose gradient centrifugation and/or the coupled peptidyltransferase assays) was recovered. However, current procedures using 12 h of centrifugation under the same conditions allow for recoveries of IF-3 as high as 85%. Additional purification of the dissociation factor activity was achieved by recentrifuging the active fractions in similar glycerol gradients but in high (0.5 M KCl) salt-containing buffers (Figure 2B). The dissociation factor sedimented somewhat slower in this gradient than in the low-salt gradient and appeared to be better resolved from the major, faster sedimenting protein peak. In both gradients, the high-molecular-weight protein resolved from IF-3 had the characteristics of ferritin. Practically all of the activity layered on the high-salt gradient was recovered in the active fractions, numbered 17–24, and the specific activity was about three times greater than that in crude extracts obtained from unresolved native subunits. When the activity obtained from the low-salt gradient (Figure 2A) was separated into the trailing (pooled fractions 21–24) and leading (pooled fractions 25–27) portions and recentrifuged individually through high-salt gradients, the position and pattern of activities obtained from the two gradients was the same.

An approximate sedimentation coefficient for the dissociation factor was obtained by centrifuging purified IF-3 in the low salt (0.05 M KCl)–glycerol gradient described above, for 12 h at 200 000*g*. Comparison of the position of IF-3, with those obtained with bovine albumin, γ-globulin, β-galactosidase, apoferritin, and RNA polymerase of *E. coli*, centrifuged in the same rotor, suggested an *s* value of 14 or 15 and a molecular weight of near 500 000. Analysis of the purified IF-3 for other components of the protein synthesizing system indicated that it was completely free of elongation factors EF-1 and EF-2, of initiation factors IF-1 and IF-2, and of the Met-$tRNA_f$ deacylase.

Polyacrylamide gel electrophoresis (under nondenaturing conditions) of the extract (Figure 3A) and of the active fractions from the low-salt (B) and high-salt (C) gradients indicated that the final preparation obtained by these procedures was at least 85% pure. Electrophoresis of the purified IF-3 in the presence of sodium dodecyl sulfate (D) revealed the presence of some 10 or 11 major polypeptide components and two or three minor bands; the subunit molecular weights ranged from 38 000 to about 135 000. The purification procedures

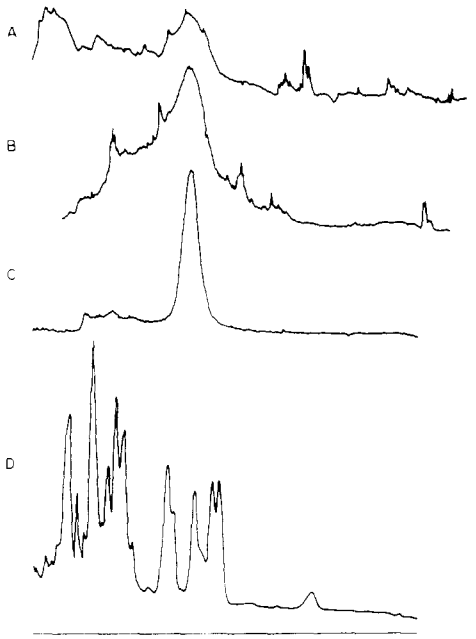

FIGURE 3: Acrylamide gel electrophoresis patterns of IF-3 containing fractions obtained from various steps in the purification procedure. (A) Native 40S extract, 52 μg of protein; (B) pooled active fractions from low (50 mM) salt glycerol gradient, 52 μg of protein; (C) pooled active fractions from high (0.5 M) salt gradient, 25 μg of protein. Samples were analyzed in 4% gels, according to the procedures described by Gabriel (1971). Pattern D was obtained with 25 μg of protein from the high (0.5 M) salt gradient, analyzed in the presence of sodium dodecyl sulfate (Weber and Osborn, 1969). The gels stained with Coomassie blue were scanned spectrophotometrically at 600 nm. The migration of the proteins in the nondenaturing gels (A, B, and C) was toward the negative pole. Migration of the polypeptides in the sodium dodecyl sulfate gel (D) was toward the positive pole. Direction of electrophoresis was toward the right.

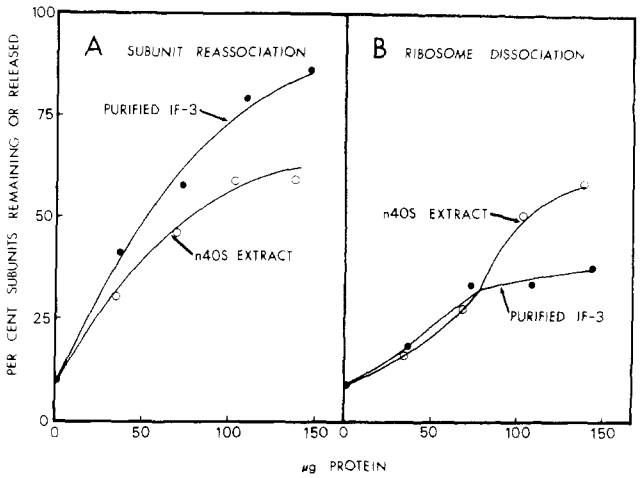

FIGURE 4: The effect of varying concentrations of dissociation factor preparations, on the interaction between derived ribosomal subunits and on the dissociation of 80S ribosomes. (A) The effect on subunit reassociation was examined by incubating d40S subunits with n40S extract (open circles) or gradient purified IF-3 (closed circles) for 20 min at 37 °C and then with d60S subunits for an additional 10 min. (B) The effect on ribosome dissociation was examined by incubating d40S with d60S subunits (to form 80S ribosomes), for 10 min at 37 °C, and then with n40S extract (open circles) or purified IF-3 (closed circles) for an additional 15 min. At the end of the incubation periods, aliquots containing 16 pmol of each of the subunits (or ribosomes) were analyzed by the sucrose gradient centrifugation assay (50 000*g*); aliquots from the same incubations, containing 8 pmol of each of the subunits, were analyzed by the coupled peptidyltransferase procedure. The results are expressed as the percent of free subunits, estimated from the ribonucleoprotein peaks in the optical density patterns, and from the concentration of 60S subunits that catalyze transpeptidation; with both assays, the values obtained were similar (Thompson et al., 1976).

described above with n40S extract have also been carried out with similar results using extracts prepared from total native subunits. Examination of the various native particles obtained by zonal centrifugation showed that all of them contained dissociation factor activity but the highest concentration was associated with the n40S subunits. Dissociation factor was not detected in the soluble supernatant of rat liver homogenates or in various ammonium sulfate fractions prepared from it; up to 2 mg of cytosol protein had no effect on ribosome dissociation or on subunit reassociation.

Resolution of the high-molecular-weight dissociation factor (IF-3) from the low-molecular-weight activities in the extract was also obtained by a variety of procedures. The addition of ammonium sulfate to 40% saturation led to the precipitation of 85–90% of the dissociation factor activity; the 40–70% ammonium sulfate fraction contained over 85% of the IF-1, IF-2, and Met-tRNA$_f$ deacylase activities. Molecular sieve chromatography of the total ribosomal subunits extract, containing IF-3, showed that 90–95% of the activity recovered was present in the void volume of Ultragel ACA 34 which excluded proteins with molecular weights of over 400 000 and was free of the other low-molecular-weight activities. Chromatography on DEAE-cellulose at pH 7.5, using elution with KCl gradients, indicated that the dissociation factor activity emerged from the column at salt concentrations near 0.15 M.

Experiments on ribosome dissociation (to release subunits from preformed 80S particles) and on subunit reassociation (to prevent formation of 80S ribosomes from subunits) were carried out with extracts obtained from pooled native subunits, from resolved native 40S subunits, and with purified IF-3 obtained from both of these starting materials. In general, the results with various preparations were quite similar. Some slight differences, however, between crude and purified dissociation factor preparations were observed. Crude extracts contained two activities that yielded slightly elevated false values for IF-3. One of these caused aggregation of 80S ribosomes, which affected gradient centrifugation analysis slightly; the decrease in 80S material with crude extracts appeared to be more drastic due to the formation of aggregates which sedimented faster than the 80S ribosomes, toward the bottom of the gradient. The other activity stimulated the transpeptidation reaction catalyzed by 60S ribosomal subunits, which affected the values obtained when incubations were analyzed with the coupled peptidyltransferase assay.

The effect of IF-3 on the dissociation of preformed 80S ribosomes appeared to be somewhat different from that on the reassociation of ribosomal subunits. Reassociation of subunits (Figure 4A) was examined by incubating varying concentrations of purified IF-3 (closed circles) or crude n40S extract (open circles) with d40S subunits, followed by a second incubation with d60S subunits before gradient centrifugation analysis. Both the purified and the crude IF-3 preparations prevented extensively the reassociation of subunits, but the specific activity of the purified IF-3 was slightly greater than that of the extract. Comparison of various preparations containing IF-3 activity indicated that the gradient purified material was usually about 30–50% more active in terms of specific activity, than the n40S extract, and two to three times more active than the extract from unresolved native subunits. The effects on the dissociation of ribosomes (Figure 4B) were examined by incubating preformed 80S ribosomes with n40S extract (open circles) or with purified IF-3 (closed circles)

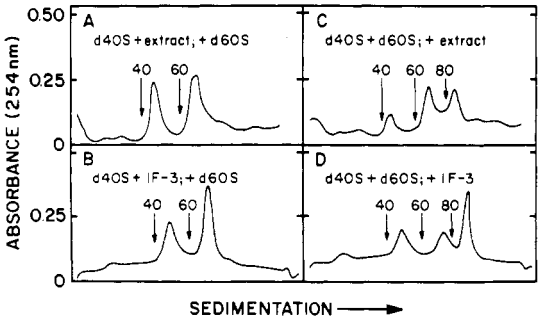

FIGURE 5: The effect of saturating concentrations of n40S extract and gradient purified IF-3 on the interaction between derived ribosomal subunits and on the dissociation of 80S ribosomes. All incubations, as described below, contained 16 pmol of d40S and d60S subunits and approximately 200 μg of n40S extract protein or gradient purified IF-3. Derived 40S subunits and n40S extract protein (A) or purified IF-3 (B) were incubated for 20 min at 37 °C and then with 16 pmol of d60S subunits for an additional 10 min. In another set of experiments, d40S and d60S subunits were incubated for 20 min at 37 °C and then with n40S extract (C) or purified IF-3 (D) for an additional 10 min. The incubations were then analyzed by centrifugation through sucrose gradients as described in the text, with the following modifications: A and C contained 5.6 mM $MgCl_2$ in the incubation and centrifugation solutions, and sedimentation was for 3.5 h at 200 000g; B and D contained 2 mM $MgCl_2$ and centrifugation was for 14 h at 50 000g.

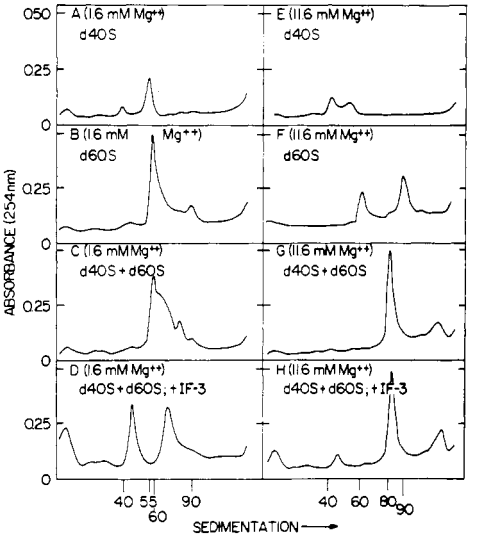

FIGURE 6: The effect of magnesium ion concentration on the interaction between derived subunits, and on the activity of dissociation factor. One set of incubations and gradient centrifugations was performed in solutions containing 1.6 mM $MgCl_2$ (A–D) and another set in 11.6 mM $MgCl_2$ (E–H). (A and E) twenty-four picomoles of d40S subunits; (B and F) 30 pmol of d60S subunits; (C and G) 24 pmol of d40S and 30 pmol of d60S subunits, incubated for 20 min at 37 °C; (D and H) 24 pmol of d40S and 30 pmol of d60S subunits, incubated for 20 min at 37 °C and then with 280 μg of n40S extract protein for an additional 10 min. The reaction mixtures were then analyzed by sucrose gradient centrifugation, as described in the text.

before gradient centrifugation. In both cases, the concentration-dependent reaction with ribosomes was less pronounced than that observed with subunits; whereas reassociation of subunits was extensively prevented (Figure 4A), active dissociation of 80S ribosomes (Figure 4B) was more limited. This can also be seen in Figure 5 which shows the gradient sedimentation patterns from incubations containing ribosomes or subunits and relatively high concentrations of IF-3 preparations. When n40S extract (Figure 5A) or gradient purified IF-3 (Figure 5B) was incubated briefly with d40S subunits, prior to the addition of 60S particles, 80S ribosomes were not formed. The peaks obtained from the gradient sedimented somewhat faster than the 40S and 60S monomers, due to the formation of complexes with dissociation factor described in more detail below. In all of the experiments described in Figure 5, resolved particles with sedimentation values of 40, 60, and 80 S were centrifuged as markers in the same rotor, in order to compare the mobilities with those of the new, faster sedimenting species. When the n40S extract or purified IF-3 was added after the d40S and d60S subunits had been allowed to form 80S ribosomes (Figures 5C and D, respectively), three ribonucleoprotein peaks were obtained from the gradient; in addition to the two peaks representing the faster sedimenting 40S and 60S particles, an additional peak (identified below as a modified 80S ribosome) was also obtained. These results suggested that complete dissociation was not obtained even when relatively high (over 200 μg of protein) concentrations of crude or purified dissociation factor preparations were used; they further suggested that ribosomal subunits were more sensitive than ribosome to the action of IF-3.

Two differences in the experients described in Figure 5 with crude extract, as compared with those with purified IF-3, should be noted. One is that two incubations (Figure 5A and C) were analyzed by gradient centrifugation for 3.5 h at 200 000g (SW 41 rotor, Spinco) and two (Figure 5B and D) for 14 h at 50 000g in the same rotor to prevent pressure-induced dissociation (Infante and Baierlein, 1971), as described below; this resulted in apparent differences in the sedimentation of the particles between the two sets. Also the experiments with purified IF-3 were carried out in solutions containing 2 mM $MgCl_2$, instead of 5.6 mM, in order to minimize the effect of Mg^{2+} on the interaction between the subunits. Thus, even at the lower concentrations of magnesium, complete dissociation was not obtained. Additional studies on the effect of varying concentrations of Mg^{2+} on the dissociation factor-catalyzed reaction were carried out (Figure 6). The sedimentation patterns of individual subunits and of combined subunits with and without n40S extract were obtained from incubations and centrifugations containing 1.6 or 11.6 mM $MgCl_2$. When incubations and centrifugations were carried out in solutions containing 1.6 mM $MgCl_2$, d40S subunits (Figure 6A) appeared mostly in the form of 55S dimers, while the 60S preparations (Figure 6B) revealed mostly monomers. When the d40S and d60S preparations were incubated together before sedimentation, a very small amount of material in the 80S region was detected (Figure 6C). Most of the ribonucleoprotein sedimented in the 55–60S region, in an unresolved form. In contrast, only an 80S peak was obtained in the control to the experiments described in Figure 5, containing d40S plus d60S particles, analyzed at markedly lower contrifugation forces (50 000g as compared with 200 000g). Some effect of n40S extract on these particles at this level of Mg^{2+}, however, was evident from the changes in sedimentation when the extract was added to the incubation containing d40S plus d60S particles (Figure 6D); subunits appeared as faster sedimenting peaks. These experiments suggested that, even at relatively low concentrations of magnesium ions, subunits reacted with IF-3 to form particle-factor complexes. The remaining portion of Figure 6 (E–H) shows similar experiments in 11.6 mM Mg^{2+}-containing solutions. Under these conditions, when d40S and d60S subunits were allowed to interact before centrifugation, most of the ribonucleoprotein was recovered as a sharp peak in the 80S region (Figure 6G); some high-molecular-weight aggregate was also obtained. When the extract was added (Figure 6H), only a small amount of material which sedimented somewhat faster than 40S and an even smaller

TABLE I: Effects of n40S Extract and Temperature of Incubation on the Reassociation of d40S Plus d60S Subunits and on the Dissociation of 80S Ribosomes, Measured by the Peptidyltransferase-Coupled Reaction.

Particles[a]	Temp (°C)[b]	Acetyl[^{3}H]Met-puromycin formed (pmol)[c]		
		− extract	+ n40S extract	Δ
d40S + d60S	2	0.72	1.83	1.11
d40S + d60S	37	0.53	2.18	1.65
80S	2	0.31	0.44	0.13
80S	37	0.30	0.88	0.58

[a] Incubations contained 8 pmol of d40S and 8 pmol of d60S subunits, or 80S ribosomes formed by preincubating similar quantities of subunits for 10 min at 37 °C. [b] Subunits or preformed ribosomes were incubated for 2 min at 2 or 37 °C, as such (−extract) or in the presence of saturating amounts (74–100 μg) of n40S extract. [c] At the end of the 2-min incubation in the presence and absence of extract, the reaction mixtures were analyzed for peptidyltransferase activity with buffered salts–ethanol, acetyl[^{3}H]methionyl-tRNA, and puromycin. The values obtained from incubations without (− extract) or with (+ n40S extract) extract and the differential between the two values (Δ) have been corrected for the amount of radioactivity extracted with ethyl acetate from duplicate incubations without puromycin.

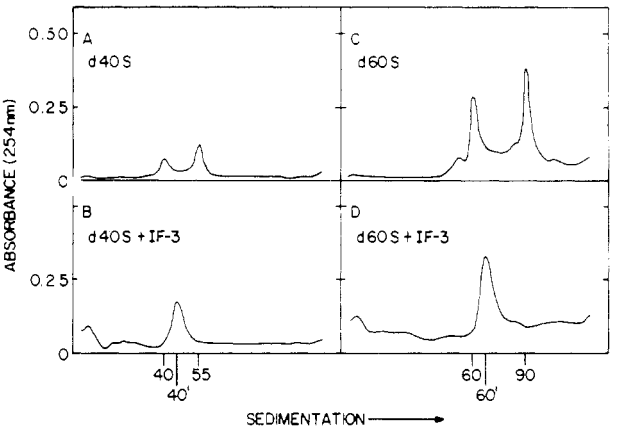

FIGURE 7: The effect of dissociation factor on the sedimentation pattern of derived subunits. (A) Approximately 16 pmol of d40S subunits; (B) 16 pmol of d40S subunits incubated with 260 μg of n40S extract protein for 30 min at 37 °C; (C) 16 pmol of d60S subunits; and (D) 16 pmol of d60S subunits, incubated with 260 μg of n40S extract protein. The reaction mixtures were then analyzed by sucrose gradient centrifugation.

amount of material slightly ahead of the 60S region were observed, but most of the 80S ribosomes remained undissociated. Thus, although IF-3 reacted to a very limited extent with 80S ribosomes (Figure 5D) and quantitatively with subunits (Figure 6D) at Mg^{2+} concentrations up to 5.6 mM, it did not affect ribosomes at relatively high magnesium ion concentrations.

Examination of the effect of temperature on the interaction between dissociation factor and ribonucleoprotein particles also indicated that the reaction with subunits was different from that with ribosomes. Preliminary experiments in which preformed 80S ribosomes were incubated with n40S extract, at varying temperatures between 2 and 37 °C, revealed that dissociation was markedly dependent on temperature; dissociation did not occur to a significant extent at temperatures below 10 °C. Table I describes an experiment in which 80S ribosomes or d40S and d60S subunits were incubated with and without n40S extract, at 2 or at 37 °C, and the reaction mixtures were then assayed for 60S-specific peptidyltransferase at 37 °C. The effect of the dissociation factor preparation on subunits, at 2 and at 37 °C, was quite similar (lines 1 and 2); relatively high levels of transpeptidation in both cases indicated that the interaction of IF-3 with subunits, preventing the formation of ribosomes, was not significantly dependent on temperature. In contrast, the dissociation factor preparation had very little effect on 80S ribosomes at 2 °C and, therefore, on the subsequent transpeptidation reaction; at 37 °C, the dissociation was increased threefold. Thus, whereas the factor-dependent reaction with subunits was only about 50% greater at 37 °C than at 2 °C, the reaction with ribosomes was four to five times more extensive at 37 °C than at 2 °C.

Similar results regarding the action on subunits as compared with ribosomes were obtained in experiments designed to examine the effect of dissociation factor concentration, not presented in detail here. Preformed 80S ribosomes or 40S and 60S subunits were incubated with n40S extract and the peptidyltransferase activity was determined. Transpeptidation was markedly stimulated when the extract was present in the incubation mixtures and, in both cases, activity was dependent on the concentration of the extract; however, subunits were more sensitive to lower concentrations of dissociation factor than preformed ribosomes. Additional sudies not shown here indicated that IF-3 reacted extensively with free (nonpolysomal) 80S particles isolated from the postmicrosomal fraction to yield subunits, but that polysome-bound ribosomes engaged in protein synthesis were resistant to dissociation factor.

As noted above (Figures 5 and 6), examination of the particles formed as a result of the interaction of IF-3 with ribosomes or ribosomal subunits revealed that the sedimentation characteristics differed from those of derived 40S and 60S particles. The effect of dissociation factor on the sedimentation of purified d40S and d60S subunit preparations is shown in Figure 7. In the absence of n40S extract, the d40S subunit preparations (Figure 7A) exhibited 40S monomer and 55S dimer peaks, and the d60S subunit preparations (Figure 7C) exhibited 60S monomer and 90S dimer peaks (Martin et al., 1971; Nonomura et al., 1971). After incubation with n40S extract, the typical monomer and dimer peaks were not present and, instead, a single peak sedimenting faster than the 40S monomer (Figure 7B, approximately 46 S, esignated as 40′ particles) and a single peak sedimenting faster than the 60S monomer (Figure 7D, approximately 69 S, designated as 60′ particles) were obtained. The conversion of the two forms of the small ribosomal subunit to 40′S particles can be seen more clearly in Figure 8. Derived 40S subunit preparations consisting of 40S monomers and 55S dimers (Figure 8A) were incubated in the presence of increasing concentrations of purified IF-3 (Figure 8B–F) before sucrose gradient centrifugation. With increasing protein concentration, the 55S peak was depressed while the material in the 40S region increased in amount and in the distance that it sedimented. It is of interest to note that the sedimentation rate of the 55S dimer remaining was also higher than that found in the absence of IF-3, suggesting that the 55S species also interacted with dissociation factor. Although not shown here, similar observations were made when IF-3 was incubated with 60S monomers plus 90S dimers but the amount of IF-3 necessary appeared to be greater than with 40S subunits.

One possible explanation for the formation of the faster sedimenting species was that they represented complexes consisting of ribosomal subunit monomer and high-molecular-weight dissociation factor. Additional characterization of

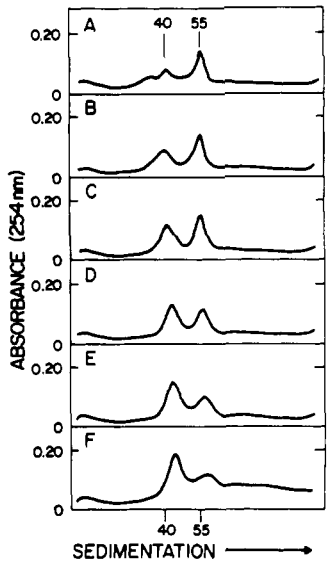

FIGURE 8: The effect of dissociation factor concentration on the monomers and dimers of ribosomal small subunits. Approximately 20 pmol of d40S subunits was incubated for 10 min at 37 °C without (A) and with 10 (B), 20 (C), 30 (D), 40 (E), or 50 (F) μg of purified IF-3. The reaction mixtures, in 4 mM $MgCl_2$, were analyzed by sucrose gradient centrifugation for 14 h at 50 000g.

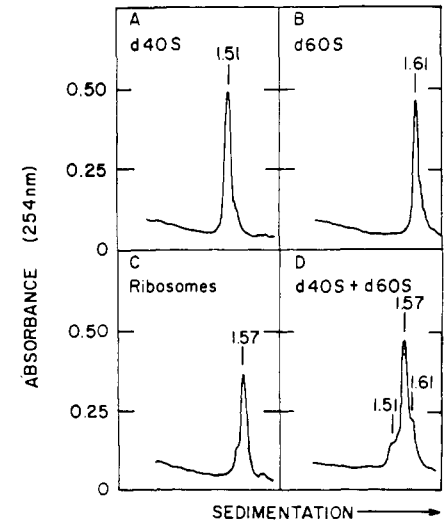

FIGURE 9: Cesium chloride density gradient centrifugation of purified ribonucleoprotein particles. (A) Approximately 16 pmol of d40S subunits; (B) 16 pmol of d60S subunits; (C) 6 pmol of ribosomes purified from microsomes by extraction with deoxycholate and 0.5 M NH_4Cl (Skogerson and Moldave, 1967, 1968); (D) 16 pmol of d40S plus 16 pmol of d60S subunits that had been incubated together for 10 min at 37 °C. Samples were fixed with formaldehyde, centrifuged in CsCl gradients, and analyzed as described by Hirsch et al. (1973) and in the text (Experimental Procedures).

the ribonucleoprotein particles and the complexes formed with unfractionated extract or with purified preparations was obtained by cesium chloride buoyant density centrifugation after formaldehyde fixation (Hirsch et al., 1973). Analysis of derived 40S subunits (Figure 9A) consisting of a mixed population of 40S monomers and 55S dimers revealed a single ribonucleoprotein peak with a density of about 1.51; the d60S monomer and d90S dimer population also gave a sharp single peak, with a buoyant density of 1.61 (Figure 9B). These values are in agreement with those of Wool and Stöffler (1974) and vary somewhat from those of Hirsch et al. (1973). Estimates calculated on the basis of the buoyant density values (Perry and Kelley, 1966) suggested that the d40S and d60S subunits contained about 55 and 39% protein, respectively. The buoyant density of polysomes (Figure 9C) purified with deoxycholate and 0.5 M NH_4Cl and that of 80S ribosomes formed from d40S plus d60S subunits (Figure 9D) was 1.57, corresponding to about 44% protein. Postmicrosomal native 40S subunits (Figure 10A) revealed a heterogeneous density distribution on CsCl gradient centrifugation, with a main peak of material at a buoyant density of about 1.41. When the n40S subunits were extracted with 0.88 M KCl and centrifuged in the CsCl gradient, the major peak obtained had a buoyant density of 1.51 (Figure 10B), the same as d40S subunits free of nonribosomal protein. Centrifugation of d40S subunits incubated with n40S extract (Figure 10C) revealed a pattern similar to that obtained with n40S subunits; although the material was heterogeneous, a considerable amount of material banded with a density of 1.41. Calculations based on the proportion of protein and RNA at a buoyant density of 1.41 suggested a structure consisting of a 40S ribosomal subunit complexed with about 700 000 daltons, on the average, of excess protein. Similar results were obtained when the analyses were carried out with particles from incubations with purified IF-3. The results were consistent with the formation of a 40S·IF-3 complex. Hirsch et al. (1973) found that native 40S subunits from ascites cells also existed as a heterogeneous population, and that those with lower densities appeared to be due to the

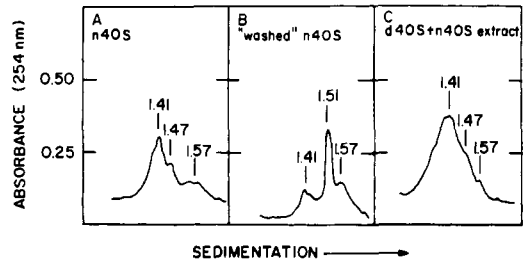

FIGURE 10. Cesium chloride density gradient centrifugation of native 40S subunits, salt-extracted n40S subunits, and reconstituted n40S subunits. The particles were fixed with formaldehyde, centrifuged on CsCl gradients, and analyzed as described above. (A) Twenty-four picomoles of n40S subunits; (B) 16 pmol of n40S subunits that had been treated with 0.88 M KCl, to obtain the n40S extract as described in Experimental Procedures; (C) 16 pmol of d40S subunits that had been incubated with 135 μg of n40S extract for 10 min at 37 °C.

association of large quantities of nonribosomal protein with the particles.

When similar analyses (not presented here) were carried out with d60S particles incubated with n40S extract or purified IF-3, the pattern obtained on CsCl gradient centrifugation revealed at least four major products, with buoyant densities of approximately 1.58, 1.51, 1.48, and 1.44. Thus, the 60S interaction with dissociation factor led to the formation of particles composed of up to 58% protein, and included minor components containing over 1×10^6 daltons excess protein. The reaction of IF-3 with d40S subunits differed from that with d60S subunits. For example, products containing more than 1×10^6 daltons excess protein were not detected in experiments with d40S subunits, even in the presence of large excesses of dissociation factor; the buoyant density and the sedimentation constant appeared to remain the same when relatively high concentrations of IF-3 were used. In contrast, increasing amounts (in excess) of dissociation factor led to an increase in the amount of protein bound to d60S particles as

evidenced by decreases in buoyant density and increases in sedimentation on sucrose gradients. Thus, it appeared that one molecule of IF-3 interacted with a 40S subunit, while more than one IF-3 could interact with d60S subunits. The characteristics of the particles formed when subunits were incubated directly with dissociation factor were compared with the particles formed when 80S ribosomes were incubated with IF-3. As shown above in Figures 5C and D, 40′S, 60′S, and a particle sedimenting faster than 80S were obtained. The particles recovered from the gradient were fixed with formaldehyde and centrifuged on CsCl gradients. The buoyant density of the 40′S particle was 1.41, while the 60′S particles consisted of several species with densities of 1.56, 1.52, 1.49, and 1.46. The observation that the 40′S and 60′S particles formed were similar, whether IF-3 was allowed to interact with subunits or with ribosomes, suggested that the dissociation factor had a common action on subunits and ribosomes. The formation of a faster sedimenting "80′S" particle was more difficult to interpret. Cesium chloride gradient analysis indicated a buoyant density of 1.57, similar to that obtained when performed 80S ribosomes were used. This finding suggested that the 80′S particle was not associated with nonribosomal protein, and could be due to a conformational change induced by the dissociation factor. This possibility is under investigation.

An additional observation has been made with particles prepared from incubations of IF-3 and d40S subunits or 80S ribosomes. The 40′S species isolated from gradients, free of unbound IF-3, did not form 80S ribosomes when incubated with d60S subunits. This finding was also consistent with the formation of a 40S·IF-3 complex, as a result of the interaction of IF-3 with the free or the ribosome-associated 40S subunit.

Discussion

It has been reported that one of the factors from ribosomal washes required for the initiation of protein synthesis with globin mRNA is a large complex that is composed of seven to ten polypeptides (Schreier and Staehelin, 1973c; Prichard and Anderson, 1974; Staehelin et al., 1975; Sundkvist and Staehelin, 1975; Freienstein and Blobel, 1975; Safer et al., 1976; Benne and Hershey, 1976). The large protein complex obtained in this laboratory from extracts of rat liver native ribosomal subunits appears to be structurally similar to this high-molecular-weight complex. The rat liver factor (IF-3) is estimated to have a molecular weight of 500 000 to 700 000 based on: (a) the coefficient of sedimentation of the purified material on glycerol gradient centrifugation; (b) the increase in sedimentation of 40S subunits when complexed to IF-3; and (c) the buoyant density of the 40S·IF-3 complex in CsCl gradients. Further, it contains about ten polypeptides resolved on sodium dodecyl sulfate gel electrophoresis; the molecular weight of the complex based on the molecular weight of its subunits (715 000) is consistent with that obtained by the other procedures. A high molecular weight for other mammalian ribosome dissociation factors is also suggested by the behavior on Sephadex G-200 (Merrick et al., 1973) and glycerol gradient centrifugation (Schreier and Staehelin, 1973a); however, values of about 100 000 (Lawford et al., 1971) and 139 000 (Nakaya et al., 1973) have been reported.

Whereas derived 40S ubunits react readily with d60S subunits to form 80S ribosomes, the native 40S particles that contain IF-3 and those formed in vitro by reaction of d40S subunits with IF-3 do not react directly with d60S particles. This observation and the finding that subunits are released from 80S ribosome in the presence of IF-3 suggest that one of the functions of this protein is that of a dissociation factor; it can bind to subunits preventing reassociation or react with ribosomes causing dissociation. Prokaryote dissociation factor IF-3 may also function by trapping free subunits in equilibrium with ribosomes (Gottlieb et al., 1975) or by actively dissociating ribosomes. The possibility that other proteins or factors may influence ribosome dissociation, as has been reported with initiation factors from *E. coli* (Godefroy-Colburn et al., 1975) is not excluded. It is of interest that both template-binding and ribosome-dissociation roles have been assigned to prokaryote IF-3 and that, in addition to the action of eukaryote IF-3 on ribosomal particles, some evidence for the role of this high-molecular-weight factor in the binding of mRNA to 40S subunits has also been reported (Staehelin et al., 1975).

The 40′S particle formed by reaction with IF-3 does not reassociate with d60S subunits due to the formation of a complex between the ribosomal subunit and the dissociation factor, and not to the inactivation of the particle. When the faster-sedimenting 40′S particle is extracted with 0.88 M KCl, the ribonucleoprotein recovered has a sedimentation value of about 40 S and a buoyant density of 1.51, characteristic of pure d40S subunits; it reacts quantitatively with d60S subunits to form ribosomes, and it can translate poly(U) in the presence of 60S subunits and the other essential componets. Also, both the native 40S and the reconstituted 40S·IF-3 complexes are capable of binding acetyl-Phe-tRNA under the appropriate conditions, such as in the presence of specific binding factors when the reconstituted complexes are used. These studies, and others in this laboratory that suggest that IF-3 is released from the 40S·IF-3 complex when it is allowed to participate in polypeptide synthesis, further indicating that the 40′S particles are functional, will be described in more detail subsequently.

The interaction between IF-3 and ribonucleoprotein particles appears to be more specific for free 40S subunits than for 60S subunits or 80S ribosomes. With 40S subunits, the reaction is quantitative and stoichiometric; that is, all of the d40S subunits are converted to a single faster sedimenting particle and complexes of 40S subunits containing more than 1 molar equiv of IF-3 have not been detected. In contrast, the IF-3 reaction with 80S ribosomes is limited in that only about half of the population appears to be dissociated; with 60S subunits, multiple complexes are found, some of which seem to contain more than 1 equiv of IF-3. The conversion of 40S monomers and dimers to 40S·IF-3 complexes occurs at lower concentrations of IF-3 than the conversion of 60S monomers and dimers to the corresponding complexes or the dissociation of ribosomes to subunits. Further, it appears that at very low concentration of IF-3, the products that are first observed to be released from 80S ribosomes include the 46S complex, 60S monomers, and 90S dimers; at higher concentrations, the "60S" products released have sedimentation values of 69 S or higher. An additional difference between subunits and ribosomes is that, whereas ribosome dissociation is markedly temperature dependent, the reaction between d40S subunits and IF-3 is not; this observation may have implications in terms of the interacting site(s) on 40S subunits in free as compared with the ribosome-associated form. It should be emphasized, however, that the results of analyses carried out with 40S·IF-3 complex that is released from 80S ribosomes are in all respects similar to those of the complex obtained when isolated d40S subunits react directly with IF-3. It has also been observed that IF-3 has no effect on template-bound ribosomes engaged in polypeptide synthesis; it does, however, dissociate part of the native 80S particle population obtained from the postmicro-

somal fraction, which may contain some "run-off" ribosomes free of template.

Benne and Hershey (1976) reported that radioactive high-molecular-weight protein prepared from reticulocyte ribosomal extracts binds to 40S subunits but not to 60S subunits; under the conditions used in this laboratory, some reaction appears to take place between IF-3 and 60S particles. It is difficult to interpret from the results presented here whether IF-3 has a specific ribosome dissociation function when bound to 60S subunits; for example, if the association is weak and non-functional, as compared with 40S·IF-3, the factor could be released from the 60S subunits and, if 40S subunits are present, react preferentially with the smaller subunits. Thus, although the final results are the same, dissociation would be primarily a consequence of the interaction between IF-3 and 40S subunits. The observation that the complexes containing 60S subunits and IF-3 readily catalyze the peptidyltransferase reaction suggests that the factor does not bind to sites that interfere with the peptidyltransferase catalytic center, or A and P substrate-binding sites, or that the factor is released under the conditions of the assay. These results also indicate that the 60S particle is not inactivated by IF-3.

Lubsen and Davis (1974) suggested that native 40S and native 60S subunits contained distinct dissociation factors. Analyses for dissociation factor activity in the several fractions obtained by zonal centrifugation of the native rat liver subunit preparations indicate that the activity is widely distributed; a relatively high concentration, however, is associated with the n40S fraction. Whereas the n40S subunits appear to be free of contamination with other particles, some material with characteristics of n40S subunits is detected in the fractions containing heavier particles. Therefore, it is difficult to determine whether dissociation factor activity in the "n60S" subunits fraction is due to the same or to a different factor, or to contamination with n40S particles. If, indeed, the dissociation factor on n40S subunits also occurs on n60S subunits, it could, to some extent, explain the interaction between IF-3 and d60S subunits detected in this laboratory. Studies are in progress to determine whether the activities on native 60S and 40S subunits are similar.

The rat liver factor purified in this laboratory appears to be active at more physiological and higher (1.6 to 5.6 mM) magnesium ion concentrations than some reported with factors from reticulocyte (Lubsen and Davis, 1972; Merrick et al., 1973; Mizuno and Rabinowitz, 1973), ascites cells (Ayuso-Parilla et al., 1973), or rat liver (Lawford et al., 1971). It should also be noted that, although a requirement for nucleotides (ATP or GTP) has been reported with some preparations of dissociation factor (Mizuno and Rabinowitz, 1973; Favelukes et al., 1973; Chen et al., 1975), the reactions described here do not require nucleotides.

In addition to IF-3, a number of low-molecular-weight proteins (under 200 000) can also be obtained from the native subunit extract by gradient centrifugation or ammonium sulfate fractionation, but these account for only a small percent of the total protein in the extract; a binding factor for Met-$tRNA_f$ that is template dependent but is GTP independent (IF-1), another that is GTP dependent but is template independent (IF-2) as reviewed by Weissbach and Ochoa (1976), and a specific Met-$tRNA_f$ hydrolase have been identified among the low-molecular-weight components and will be described subsequently. Comparison of the polypeptides obtained on sodium dodecyl sulfate gel electrophoresis of IF-3 with those of the two initiator Met-$tRNA_f$ binding factors did not reveal homologies.

The results presented here are consistent with a ribosome cycle in which subunits participate as intermediates. Thus, at the termination of protein synthesis, ribosomes are (a) released as such from polysomes and are dissociated by a protein factor into subunits, (b) are released from polysomes as subunits and prevented by dissociation factor from reassociating, or (c) are released as ribosomes which spontaneously dissociate into subunits and then react with dissociation factor. The exact mechanism by which subunits are formed is unknown; however, regardless of sequence the intermediate appears to be a factor-associated subunit which may have other functions but which is subsequently processed for interaction with other components when it participates in peptide chain initiation and synthesis. Thus, some of the n40S subunits represent a transient intermediate in the ribosome cycle, composed of 40S particles with IF-3 and other protein factors.

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