Nat. Acad. Sci. U. S. 68, 1331.
Mendecki, J., Lee, S. Y., and Brawerman, G. (1972), *Biochemistry 11*, 792.
Miller, R. L., and Plagemann, P. G. W. (1972), *J. Gen .Virol. 17*, 349.
Noller, C. R. (1965), The Chemistry of Organic Compounds, 3rd ed, Philadelphia, Pa., W. B. Saunders, p 426.
Penman, S., Fan, H., Penman, S., Robash, M., Weinberg, R., and Zylber, E. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35*, 561.
Penman, S., Vesco, C., and Penman, M. (1968), *J. Mol. Biol. 34*, 49.
Perry, R. (1963), *Exp. Cell Res. 29*, 400.
Perry, R. P., and Kelley, D. (1970), *J. Cell Physiol. 76*, 127.
Perry, R. P., LaTorre, J., Kelley, D. E., and Greenberg, J. R. (1972), *Biochim. Biophys. Acta 267*, 220.
Philipson, L., Wall, R., Glickman, G., and Darnell, J. E. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2806.
Roberts, W. K. (1965), *Biochim. Biophys. Acta 108*, 474.
Roberts, W. K., and Coleman, W. H. (1971), *Biochemistry 10*, 4304.
Roberts, W. K., and Coleman, W. H. (1972), *Biochem. Biophys. Res. Commun. 46*, 206.
Roberts, W. K., and D'Ari, L. (1968), *Biochemistry 7*, 592.
Roberts, W. K., and Newman, J. F. E. (1966), *J. Mol. Biol. 20*, 63.
Roberts, W. K., Newman, J. F. E., and Rueckert, R. R. (1966), *J. Mol. Biol. 15*, 92.
Roberts, W. K., and Quinlivan, V. D. (1969), *Biochemistry 8*, 288.
Rosenfeld, M. G., Abrass, I. B., and Perkins, L. A. (1972), *Biochem. Biophys. Res. Commun. 49*, 230.
Schultz, G., Beato, M., and Feigelson, P. (1972), *Biochem. Biophys. Res. Commun. 49*, 680.
Schultz, G., Manes, C., and Hahn, W. E. (1973), *Develop. Biol.* (in press).
Sheldon, R., Jurale, C., and Kates, J. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 417.
Siegel, M. (1968), *Compr. Biochem. 26*, 1.
Sullivan, N. L., and Roberts, W. K. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1302.
Yogo, Y., and Wimmer, E. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1877.

Two Physically and Functionally Distinct Forms of Eukaryotic 40S Ribosomal Subunits†

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ABSTRACT: Preparations of the small subunit of rat liver ribosomes made in buffer containing puromycin and high concentrations of potassium (0.8 M) contain two forms of the particle; one of the two species sediments as a dimer in buffers having low concentrations of potassium (80 mM) and the other as a monomer. The two forms of the 40S ribosomal subunit are not in equilibrium; rather they remain physically distinct when sedimented through several cycles of buffers containing high and low concentrations of potassium. The monomer ($40S_m$) always has a sedimentation coefficient of 40; the dimer ($40S_d$) sediments at 40 S in high concentrations of potassium but at 55 S in low concentrations. The predominant RNA of both particles is 18S ribosomal RNA, and they have the same set of ribosomal proteins; electron microscopy confirmed that one is a dimer of the other. The buoyant density of the monomer is 1.522 g/cm^3, whereas that of the dimer is 1.514 g/cm^3. The difference in buoyant density suggested the former might contain more RNA than the latter, and indeed each $40S_m$ particle was found to have a molecule of deacylated tRNA associated with it, whereas only a small percentage of the $40S_d$ species had deacylated tRNA bound to them. The $40S_d$ species was more active than the $40S_m$ particle in the binding of aminoacyl-tRNA in the reaction catalyzed by eukaryotic initiation factor 1 (EIF-1). The dimers were also more active when assayed with an excess of 60S subunits: in the synthesis of polyphenylalanine at 12 mM magnesium (when only eukaryotic elongation factors 1 and 2 are required) as well as at 3.5 mM (when EIF-1 and EIF-2 are needed also), and in the translation of encephalomyocarditis RNA.

The small subparticles of prokaryotic and eukaryotic ribosomes can occur *in vitro* as either monomers or dimers (Pestka and Nirenberg, 1966; Tashiro and Morimoto, 1966; Igarashi and Kaji, 1969; Terao and Ogata, 1970; Petermann and Pavlovec, 1971; Martin *et al.*, 1971; Nonomura *et al.*, 1971). Whether dimers are formed *in vivo* as well is not known. The physical basis for the dimerization of ribosomal subparticles is not certain either, although electrostatic forces and divalent salt bridges are likely to play a role since dimers occur more readily in dilute than in concentrated potassium chloride buffers (Igarashi and Kaji, 1969; Petermann and Pavlovec, 1971) and dimerization is dependent on the magnesium concentration (Pestka and Nirenberg, 1966; Tashiro and Morimoto, 1966; Petermann and Pavlovec, 1971; Zamir *et al.*, 1971). It is possible that particular ionic conditions favor a conformation which allows dimerization (Zamir *et al.*, 1971). It is not known if monomers and dimers of small ribosomal subunits are in equilibrium, or

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whether the two forms are physically distinct and hence not easily interconverted.

There are conflicting reports on the relative activities of small subunit monomers and dimers. Pestka and Nirenberg (1966) found that the binding of poly(U)[1] and of phenylalanyl-tRNA was preferentially if not exclusively to the dimer of *Escherichia coli* 30S subunits. Zamir *et al.* (1971), on the other hand, report that only the inactive form of the 30S particle dimerizes and that dimers do not form couples with the large ribosomal subunit. Igarashi and Kaji (1969) confirm the latter: binding of phenylalanyl-tRNA, in their experiments, was to 30S monomers; dimers were inactive or at best the binding was weak.

The small subunit of rat liver ribosomes occurs in two forms (Henshaw and Loebenstein, 1970) which can be distinguished by their buoyant density (1.52 and 1.45 g/cm^3). The latter particles have extra proteins which may be initiation factors. Ceccarini *et al.* (1970) have observed a 50S particle in the postmitochondrial supernatant from *Dictyostelium purpureum*. The particle is likely to be a dimer of the 40S subunit since it also contains 17S RNA (as does the 40S subparticle) and has the same buoyant density. However, the 50S particle, unlike 40S subunits, is not labeled during a 5-min pulse with [^{32}P]orthophosphate or [^{3}H]uridine, although it is if incubation with the radioactive precursor is for a longer period. The observations suggest a difference in the function of the 40S and 50S forms of the small subunit of *Dictyostelium purpureum* ribosomes. Mullock *et al.* (1971) have found two forms of the small subunit of rat liver ribosomes (but, interestingly, not of hepatoma ribosomes) both of which contain 18S RNA; one, however, has a sedimentation coefficient of 45, the other of 35. When the ribosomal RNA is labeled with [^{14}C]orotic acid or [^{32}P]orthophosphate, the specific activity of the 45S particle is greater than that of the 35S species. The small subunit of Chinese hamster cell ribosomes sediments in two forms; both contain 18S RNA; however, the slower sedimenting particle contains 12S RNA (perhaps, mRNA) in addition (Enger and Walters, 1970). It is unlikely that the several types of eukaryotic small ribosomal subunit heterogeneity (dimerization, difference in buoyant density, difference in sedimentation) have a common basis. The various forms of the subparticle may be the result of a fraction of the subunits having one or another of the factors (initiation proteins, tRNA, mRNA, etc.) required for protein synthesis still associated with them and, thereby, differing from vacant ribosome subunits. It is also possible that vacant ribosome subunits can exist in physically and functionally distinct forms.

We undertook to confirm the existence of dimers of the small subparticle of eukaryotic ribosomes. Having accomplished that we studied the circumstances in which dimers occurred, devised means of isolating and purifying them, analyzed their chemical and physical properties, and, finally, compared their activity with monomers of the small subunits.

Materials and Methods

Materials. Puromycin dihydrochloride was purchased from Nutritional Biochemicals Corporation, bentonite from Fisher Scientific Co., poly(uridylic acid) from Miles Laboratories, unfractionated *Escherichia coli* B tRNA from Schwarz/Mann, and [^{3}H]phenylalanine (5 Ci/mmol) and a mixture of 15 tritiated L-amino acids (1 mCi/ml) from New England Nuclear Corporation. [^{3}H]Phe-tRNA refers to *Escherichia coli* B tRNA aminoacylated with [^{3}H]phenylalanine and 19 nonradioactive amino acids (von Ehrenstein and Lipmann, 1961; Wool and Cavicchi, 1967).

Media. The following media were used: A, 50 mM Tris-HCl (pH 7.6)–80 mM KCl–12.5 mM $MgCl_2$–10 mM HSEtOH; B, 10 mM Tris-HCl (pH 7.6)–120 mM KCl–3.5 mM $MgCl_2$–10 mM HSEtOH; C, 50 mM Tris-HCl (pH 7.6)–880 mM KCl–12.5 mM $MgCl_2$–10 mM HSEtOH; D, 10 mM Tris-HCl (pH 7.6)–80 mM KCl–5 mM $MgCl_2$–10 mM HSEtOH; E, 20 mM Tris-HCl (pH 7.6)–125 mM KCl–5 mM $MgCl_2$–10 mM HSEtOH.

Preparation of Ribosomal Particles. Liver ribosomes were isolated from male Sprague-Dawley rats that weighed 100–120 g (Martin and Wool, 1968, 1969). Ribosomal subunits were prepared by suspending ribosomes (approximately 4.5 mg of rRNA/gradient) in medium C containing 0.1 mM puromycin and incubating for 15 min at 37°; the subunits were separated on linear sucrose gradients (Martin and Wool, 1968, 1969; Stirewalt *et al.*, 1971). Fractions containing the ribosomal subunits were displaced from the gradients with 50% sucrose using an Instrument Specialities Co., Inc. (ISCO) density gradient fractionator, and the effluent was analyzed at 254 nm with an ISCO Model UA-2 uv analyzer. Fractions collected from the gradients were dialyzed overnight against medium A and the subunits were precipitated with 0.2 of a volume of ethanol (Kaulenas, 1971). The concentration of ribosomes and ribosomal subunits was calculated from the absorption at 260 nm (Wool and Cavicchi, 1966); 1 A_{260} unit was taken to be the equivalent of 45 μg of rRNA.

When large amounts of ribosomal subunits were required they were prepared in a Spinco Ti 15 zonal rotor. Ribosomes (500–675 mg of rRNA) were suspended in medium C and incubated for 15 min at 37° with 0.1 mM puromycin; separation was on a hyperbolic sucrose density gradient (Eikenberry *et al.*, 1970) as described by Sherton and Wool (1972).

The preparation of monomers (40S) and dimers (55S) of the small ribosomal subunit was as follows. The ethanol-precipitated 40S ribosomal subunits, collected from preparative sucrose gradients (*cf.* above and Figure 1a), were suspended in medium D and incubated for 5 min at 37° to dissociate aggregates. The particles (0.9–2.7 mg of rRNA) were layered on a 10–30% sucrose gradient in medium D and centrifuged at 27,000 rpm for 5 hr at 28° in Spinco SW-27 rotor. The 40S monomer ($40S_m$) and dimer ($40S_d$) fractions were collected (Figure 1b), dialyzed overnight against medium D, and precipitated with ethanol.

Analysis of Ribosomal RNA. Ribosomal RNA and protein were dissociated with sodium dodecyl sulfate (Gilbert, 1963). Ribosomes or ribosomal subunits were suspended in 50 mM Tris-HCl (pH 7.5) containing 0.5% sodium dodecyl sulfate, incubated for 5 min at 37°, and layered on a 5–20% sucrose gradient in 50 mM Tris-HCl (pH 7.5). Centrifugation was at 60,000 rpm for 100 min at 4° in a Spinco SW-65 rotor. The distribution of RNA in the gradient was determined with an ISCO density gradient fractionator and uv analyzer.

Determination of the Buoyant Density of Ribosomal Particles. Ribosomal particles were fixed by treatment with an equal volume of 8% formaldehyde in 0.25 M $NaHCO_3$ (pH 7.2) for 1 hr at 0° (Spirin *et al.*, 1965). The fixed particles (18 μg of rRNA) were layered on preformed CsCl gradients (ρ

[1] Abbreviations used are: poly(U), poly(uridylic acid); [^{3}H]Phe-tRNA, unfractionated *Escherichia coli* B transfer ribonucleic acid aminoacylated with [^{3}H]phenylalanine and 19 nonradioactive amino acids; HSEtOH, 2-mercaptoethanol; EMCV, encephalomyocarditis virus; $40S_m$, the form of the small ribosomal subunit that sediments as a monomer in 80 mM potassium chloride and 5 mM magnesium chloride; $40S_d$, the form that sediments as a dimer; EF-1 and -2, eukaryotic elongation factors (formerly called aminoacyltransferase I and II); EIF-1, -2, and -3, eukaryotic initiation factors.

1.47–1.57 g/cm^3) in buffer (20 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$). Centrifugation was at 40,000 rpm for 16 hr at 4° in a Spinco SW-50.1 rotor. The buoyant density of the particles was calculated from the refractive index at 25° (Ifft *et al.*, 1961).

Analysis of Ribosomal Proteins by Two-Dimensional Polyacrylamide Gel Electrophoresis. Proteins were extracted from preparations of ribosomal subunits with acetic acid by a modification (Sherton and Wool, 1972) of a procedure described by Hardy *et al.* (1969). The proteins were separated by two-dimensional polyacrylamide gel electrophoresis (Kaltschmidt and Wittmann, 1970; Sherton and Wool, 1972).

Assay of the Binding of Phe-tRNA to 40S Ribosomal Subunits. The extent to which 40S ribosomal subunits would bind Phe-tRNA in the reaction catalyzed by EIF-1 was determined as described by Leader *et al.* (1970) except that the assay was in medium B.

Measurement of Polyphenylalanine Synthesis. For the measurement of the synthesis of polyphenylalanine, ribosomal subunits were incubated for 10 min at 37°. Synthesis was assayed in two different circumstances: assay I (at 12 mM $MgCl_2$ without addition of initiation factors), 2.15 μg (rRNA) of 40S and 10.72 μg of 60S subunits were incubated in 0.1 ml of buffer (10 mM Tris-HCl (pH 7.6)–120 mM KCl–12 mM $MgCl_2$–10 mM HSEtOH) containing 50 nmol of GTP, 100 nmol of ATP, 180 μg of creatine phosphate, 20 μg of creatine phosphate kinase, 10 μg of poly(U), 60 μg of [^{3}H]Phe-tRNA (45,000 cpm), and 0.28 mg of G-25 fraction protein (Leader *et al.*, 1970); assay II (at 5 mM $MgCl_2$ with addition of initiation factors), 2.15 μg (rRNA) of 40S and 10.72 μg of 60S subunits were incubated in 0.1 ml of medium E containing 10 nmol of GTP, 100 nmol of ATP, 180 μg of creatine phosphate, 20 μg of creatine phosphate kinase, 100 μg of poly(U), 0.5 mmol of [^{3}H]phenylalanine (5 Ci/mmol), 5 mmol each of 19 nonradioactive amino acids, and 375 μg of a G-25 fraction protein from Krebs ascites tumor cell cytosol (Mathews and Korner, 1970) which contains initiation and elongation factors (Leader *et al.*, 1972).

Protein synthesis was terminated by the addition of 1 ml of 10% trichloroacetic acid and the samples were heated at 90–95° for 15 min and then cooled on ice. The precipitate was collected on Reeve Angel filters and washed with 30 ml of 5% trichloroacetic acid containing 10 μg/ml of phenylalanine. The filters were dried and placed into glass vials containing scintillation fluid (Wool and Cavicchi, 1967). Radioactivity was determined in a Packard Tri-Carb spectrometer; the efficiency of the determination of the radioactivity was 16%.

Measurement of the Translation of EMCV RNA. Encephalomyocarditis virus of the K2 strain was grown on Krebs II ascites tumor cells (Bellett and Burness, 1963); the virus was purified (Aviv *et al.*, 1971) and the RNA extracted (Kerr *et al.*, 1966; Mathews and Korner, 1970). For the assay of the translation of EMCV RNA ribosomal subunits (1.27 μg (rRNA) of 40S and 5.35 μg of 60S) were incubated for 45 or 30 min at 37° in 0.05 ml of medium E containing 5 nmol of GTP, 50 nmol of ATP, 90 μg of creatine phosphate, 10 μg of creatine phosphate kinase, 0.25 nmol of [^{3}H]phenylalanine (5 Ci/mmol), 2.5 mmol of each of 19 nonradioactive amino acids, 0.375 mg of G-25 fraction protein from Krebs ascites tumor cell cytosol, and 6.6 μg of EMCV RNA.

The reaction was stopped by addition of 2 ml of 10% trichloroacetic acid and the radioactivity incorporated into protein was determined as described above for the measurement of the synthesis of polyphenylalanine.

Preparation of Low Molecular Weight RNA from Ribosomes. Ribosomes (1.35–4.5 mg of rRNA) were suspended in medium D supplemented with 0.06 vol of 0.1 M EDTA (pH 7.0), 0.12 vol of 5% sodium deoxycholate, and 0.1 vol of 6% bentonite. Low molecular weight RNA was extracted from the ribosomes with phenol and 1 M NaCl by the method used by Mosteller *et al.* (1968); a second phenol extraction was carried out before the salt treatment to ensure the removal of all protein. We estimated the efficiency of the extraction of low molecular weight RNA from ribosomes to be about 55%. The estimate was made from a calculation of the recovery by the same procedure of a known quantity of 4S transfer RNA from a mixture containing ribosomal RNA.

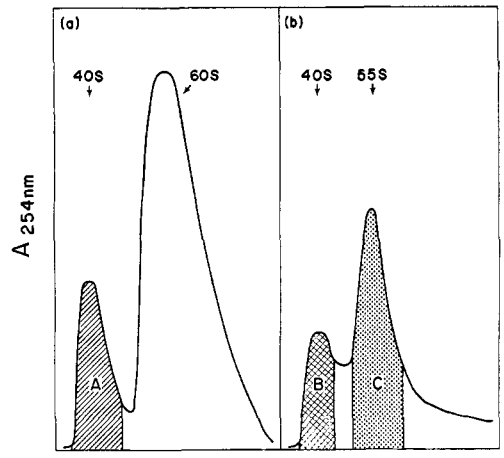

FIGURE 1: Preparation of ribosomal subunits by zonal sedimentation. (a) Ribosomes were suspended in medium C containing 0.1 mM puromycin and incubated for 15 min at 37°. The suspension (containing 4.5 mg of rRNA) was layered on a 10–30% linear sucrose gradient in medium C and centrifuged at 27,000 rpm for 4 hr at 25° in a Spinco SW-27 rotor. The 40S subunits (shaded area A) were collected, dialyzed overnight against medium A, and precipitated with ethanol. (b) The 40S subunits (1.8 mg of rRNA) were suspended in medium D, incubated for 5 min at 37° to reduce the amount of aggregation, and layered on 10–30% linear sucrose gradients in medium D; centrifugation was at 27,000 rpm for 5 hr at 28° in a Spinco SW-27 rotor. The 40S subunit monomers ($40S_m$) (shaded area B) and dimers ($40S_d$) (shaded area C) were collected.

Measurement of the Capacity of Low Molecular Weight RNA to Accept Amino Acids. The low molecular weight RNA extracted from 40S ribosomal subunits was assayed for its capacity to be aminoacylated (Dube *et al.*, 1969). The reaction mixture (0.1 ml) contained, in addition to the low molecular weight RNA to be assayed or a standard preparation of tRNA, the following: 0.85 μmol of Tris-HCl (pH 7.5), 1 μmol of KCl, 0.65 μmol of magnesium acetate, 0.3 μmol of ATP, 7 nmol of GTP, 0.4 nmol of each of 20 L-amino acids, 2 μl of a mixture of 15 tritiated L-amino acids (1 mCi/ml), and 30 μl of a preparation of rat liver aminoacyl-tRNA synthetases (see below). Incubation was for 15 min at 37°. The reaction was stopped by adding 2 ml of 2 N HCl; the precipitate was collected on glass fiber disks (Whatman GF/C) and washed several times with 5% trichloroacetic acid. The disks were dried and the radioactivity was determined as described above for measurement of the synthesis of polyphenylalanine.

Aminoacyl-tRNA synthetases were prepared from the supernatant obtained by centrifugation of a rat liver homogenate at 100,000*g* (Martin and Wool, 1969). The supernatant was treated with ammonium sulfate and the proteins precipitating between 30 and 70% saturation were collected by centrifugation, dissolved in buffer (10 mM $KHPO_4$ (pH 6.5)–250 mM KCl–10 mM HSEtOH–10% glycerol), and di-

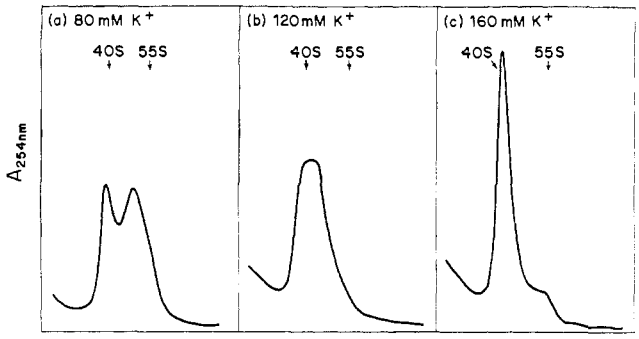

FIGURE 2: Effect of the concentration of potassium on the sedimentation of the small ribosomal subunit. Preparations of small ribosomal subunits (about 6 μg of rRNA) were suspended in buffer (10 mM Tris-HCl (pH 7.6)–10 mM $MgCl_2$–10 mM HSEtOH) containing the concentration of KCl specified. The suspension was incubated for 5 min at 37° and layered on a 10–30% sucrose gradient in the same buffer and centrifuged at 60,000 rpm for 50 min at 28° in a Spinco SW-65 rotor.

alyzed against the same buffer for 6 hr. The dialyzed preparation was mixed with DEAE-cellulose to remove residual tRNA; the DEAE-cellulose was removed by centrifugation. The treatment was repeated and the combined supernatants were dialyzed for 4 hr against 50 mM Tris-HCl (pH 7.5) containing 20 mM HSEtOH; an equal volume of 100% glycerol was added and the aminoacyl-tRNA synthetase preparation was stored at −20°. The enzyme preparation had no endogenous amino acid acceptor activity.

Rat liver tRNA to be used as a standard in the reaction was prepared from a 100,000*g* supernatant in the same way that low molecular weight RNA was prepared from ribosomes (see above). The tRNA was deacylated by the method described by Mosteller *et al.* (1968).

Results

Isolation and Characterization of Two Forms of Eukaryotic 40S Ribosomal Subunits. Ribosomes from rat liver are dissociated into subparticles when treated with puromycin and high concentrations of potassium chloride (Martin and Wool, 1969; Stirewalt *et al.*, 1971). The small ribosomal subunits prepared in that way sedimented as a single species when centrifugation was in 0.8 M potassium chloride and 12.5 mM magnesium chloride (Figure 1a); however, if the particles were analyzed in sucrose gradients containing 80 mM potassium (and 5 mM magnesium), two species, having sedimentation coefficients of 40 and 55, were observed (Figure 1b). The 55S particle, which we had seen before (Martin *et al.*, 1971), was presumed to be a dimer of the small ribosomal subunit (Tashiro and Morimoto, 1966; Petermann and Pavlovec, 1971). We shall provide evidence for that presumption below. For convenience we shall refer to the monomer as the $40S_m$ fraction and the dimer as the $40S_d$ fraction.

Effect of Cations. Since the dimerization of the small ribosomal subunit seemed to be conditioned by the concentration of ions we undertook to study that parameter with more precision. As the concentration of potassium was raised (the concentration of magnesium was kept at 10 mM) 55S dimers were converted to 40S monomers (Figure 2). The critical concentration of potassium appeared to be between 120 and 180 mM—certainly less than the 0.8 M used in preparing the subparticles.

The amount of the 55S species in sucrose gradients was decreased when the magnesium concentration was raised from 5 to 15 mM (the potassium concentration was kept at 80 mM). However, the loss of the 55S particle was not accompanied by an increase in the $40S_m$ species (results not shown). We inferred that the dimer aggregated in 15 mM magnesium and was sedimented during centrifugation, thereby leading to a decrease in the amount of material contained in the gradient. The differential sensitivity of the two forms of the small subunit to high concentrations of magnesium suggested they might be physically distinct particles.

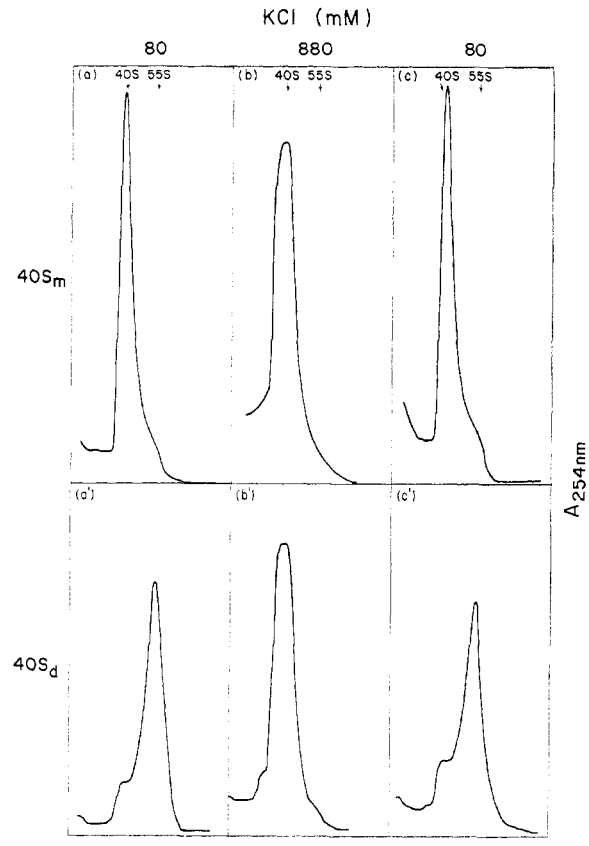

FIGURE 3: Sedimentation of small ribosomal subunits: recycling of $40S_m$ and $40S_d$ subunits in 80 and 880 mM potassium. Small ribosomal subunits were prepared by treatment with puromycin in medium C (as in Figure 1a) and separated into $40S_m$ and $40S_d$ fractions by centrifugation in medium D (as in Figure 1b). Approximately 11 μg (rRNA) of $40S_m$ and $40S_d$ particles were analyzed on 10–30% sucrose gradients in medium D (without HSEtOH); centrifugation was at 60,000 rpm for 50 min at 28° in a Spinco SW-65 rotor (a and a'). An additional 135 μg (rRNA) of $40S_m$ and of $40S_d$ fractions from the same preparations were incubated separately for 15 min at 37° in medium C containing 0.1 mM puromycin and layered on 10–30% sucrose gradients in medium C and centrifuged at 60,000 rpm for 50 min at 28° in a Spinco SW-65 rotor (b and b'). The ribosomal particles from b and b' were collected, dialyzed for 3 hr against medium A, and precipitated with ethanol; 10 μg (rRNA) of $40S_m$ and $40S_d$ fractions was analyzed on 10–30% sucrose gradients in medium D (without HSEtOH); centrifugation was at 60,000 rpm for 50 min at 28° in a Spinco SW-65 rotor (c and c').

Sedimentation. We wished to test whether $40S_m$ and $40S_d$ fractions were in equilibrium. The two species were isolated (Figure 1b) and recentrifuged (Figure 3) in medium D (which contains 80 mM KCl). The particles in the $40S_m$ fraction sedimented as a single symmetrical peak of 40 S (Figure 3a); the particles in the $40S_d$ fraction sedimented mainly at 55S with a small shoulder at 40S (Figure 3a'). Neither the $40S_m$ nor the $40S_d$ fraction reproduced the sedimentation pattern of the

TABLE I: Binding of Phe-tRNA to $40S_m$ and $40S_d$ Ribosomal Subunits.

Subunit	Phe-tRNA Bound (pmol)[a]
$40S_m$	0.56
$40S_d$	1.30

[a] Ribosomal subunits (4.28 μg (rRNA) of $40S_m$ or $40S_d$) were incubated for 15 min at 30° in 0.1 ml of medium B containing 40 μg of [^{3}H]Phe-tRNA (30,000 cpm), 10 μg of poly(U), 25 nmol of GTP, and 0.28 mg of the G-25 fraction protein which contains EIF-1 (Leader *et al.*, 1972). The [^{3}H]Phe-tRNA bound to ribosomal subunits was collected on nitrocellulose filters and the radioactivity determined with an efficiency of 16%; thus, 1000 cpm was the equivalent of 0.57 pmol of Phe-tRNA bound to ribosomal subunits. Binding was dependent on the G-25 fraction protein and on template (poly(U)).

TABLE II: Synthesis of Polyphenylalanine.

Assay	Phenylalanine Polymerized (pmol)[a] $40S_m$	$40S_d$
I	5.59	18.01
II	8.82	14.96

[a] The synthesis of polyphenylalanine by ribosomes reconstituted from subunits was assayed in two circumstances: assay I, at 12 mM magnesium with the rat liver cytosol G-25 fraction protein; assay II, at 5 mM magnesium with the ascites tumor cell cytosol G-25 fraction protein. The exact conditions are given in Methods. The molar ratio of 60S:40S ribosomal subunits was 1.7. Each 1000 cpm of radioactivity was the equivalent of the incorporation of 0.57 pmol of phenylalanine into protein.

original preparation of small ribosomal subunits (Figure 1b); thus it is unlikely the two species are in equilibrium. Some dissociation of dimers may occur due to pressure generated during centrifugation (Infante and Krauss, 1971) and could account for the small amount of 40S monomers observed when $40S_d$ fractions were analyzed (Figure 3a'). Indeed, the proportion of $40S_m$ and $40S_d$ fractions was affected by the speed of centrifugation. For example, when a preparation of small ribosomal subunits was centrifuged for 4 hr at 27,000 rpm (95,100*g*) in a Spinco SW-27 rotor 35% of the particles were $40S_m$; when centrifugation was for 40 min at 60,000 rpm (257,600*g*) in a Spinco SW-65 rotor 45% of the particles were $40S_m$. The increase in 40S monomers we presume to be due to the greater pressure with the higher speed of centrifugation.

It seemed certain that $40S_m$ and $40S_d$ particles were physically distinct, but we could not be sure that the distinction was not a result of our manipulations during their preparation. In particular we wished to test whether exposure to high concentrations of potassium, or to puromycin, or precipitation with ethanol had produced some irreversible change in a portion of the particles. For that purpose recycling experiments were carried out (Figure 3). The two species of small ribosomal subunits—$40S_m$ and $40S_d$—were isolated (Figure 1b) and then analyzed separately in sucrose gradients containing 80 mM potassium (Figure 3a and a'), reisolated and analyzed at 880 mM potassium (Figure 3b and b'), and isolated once again and analyzed finally at 80 mM potassium (Figure 3c and c'). It is clear that the conversion of the $40S_d$ fraction to $40S_m$ which occurs in high concentrations of potassium (Figure 3b') is reversible (Figure 3c'). The particles sediment true: $40S_d$ particles always sediment at 40 S when the potassium concentration is high but always at 55 S when it is low; the $40S_m$ particles sediment at 40 S no matter what the potassium concentration is. The results make it unlikely that the difference in sedimentation is due to a component of the particles that can be added and removed.

The Function of $40S_m$ and $40S_d$ Particles. We undertook to determine the function of $40S_m$ and $40S_d$ particles by assessing their capacity to participate in several of the partial reactions of protein synthesis. The two particles did differ in function: the $40S_d$ species was the more active.

Binding of Phe-tRNA. The initiation factor EIF-1 (formerly called the 40S binding factor (Leader *et al.*, 1970)) catalyzes the template specific binding of Phe-tRNA to 40S ribosomal subunits. In that reaction $40S_d$ particles bound 2.3 times as much Phe-tRNA as did an equimolar amount of $40S_m$ particles (Table I). Approximately 20% of the $40S_d$ particles bound Phe-tRNA, whereas only 8% of the $40S_m$ particles were active.

Synthesis of Polyphenylalanine. The ability of the two species of 40S particles to participate in the synthesis of polyphenylalanine was determined in the presence of an excess of 60S subunits. The assay was carried out in two different circumstances (Table II). No matter what the conditions were, the synthesis of polyphenylalanine was always greater if $40S_d$ subunits were used, although the extent of the difference did depend on the conditions. At lower concentrations of magnesium (3.5 mM) optimum synthesis of polyphenylalanine requires the initiation factors EIF-1 and -2 as well as elongation factors EF-1 and -2 (Shafritz and Anderson, 1970), whereas at higher concentrations of magnesium (12 mM) only elongation factors are required. The synthesis of polyphenylalanine was 1.7 times greater with $40S_d$ subunits than with $40S_m$ subunits when the measurement was at 3.5 mM magnesium in the presence of the two initiation factors (assay II, Table II) and 3.2 times greater at 12 mM magnesium (assay I, Table II).

Translation of EMCV RNA. In the proper conditions eukaryotic ribosomes will translate EMCV RNA with fidelity, for virus-specific peptides are synthesized *in vitro* (Mathews and Korner, 1970); translation requires all three initiation factors (Leader *et al.*, 1972). The translation of EMCV RNA was more efficient if $40S_d$ subunits were used (Table III); the difference was small (from 11 to 40%). It is perhaps significant that the difference was greater after 30 min (40%) than after 45 (11 and 20%). One interpretation of the time dependence of the difference in translation of EMCV RNA by ribosomes formed from $40S_d$ and $40S_m$ subunits is that the latter are slowly activated during the incubation (*cf.* Zamir *et al.*, 1971).

Chemical and Physical Properties of $40S_m$ and $40S_d$ Subunits. Having established that the population of small ribosomal subunits contained physically and functionally distinct particles we wished to determine if there was a chemical basis for the difference.

Buoyant Density. If $40S_m$ and $40S_d$ differ in chemical composition (*i.e.*, in the absolute amount of RNA or protein) then their buoyant densities should differ. The buoyant density of the $40S_d$ species was 1.514 g/cm^3, significantly lower than the density (1.522 g/cm^3) of the $40S_m$ particles (Table IV). The

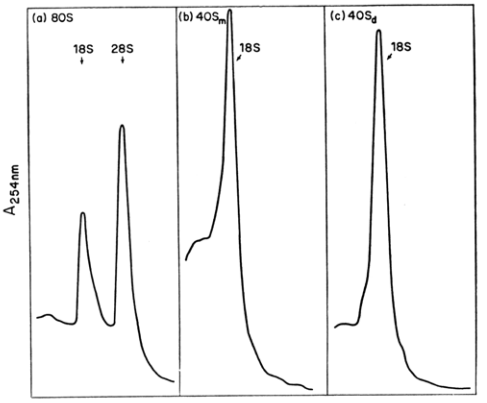

FIGURE 4: Analysis of the RNA from ribosomes and from 40S$_m$ and 40S$_d$ particles. Ribosomal RNA and protein were dissociated with sodium dodecyl sulfate and the samples (containing 9 μg of rRNA) were layered on 5–20% sucrose gradients in 50 mM Tris-HCl (pH 7.5). Centrifugation was at 60,000 rpm for 100 min at 4° in a Spinco SW-65 rotor.

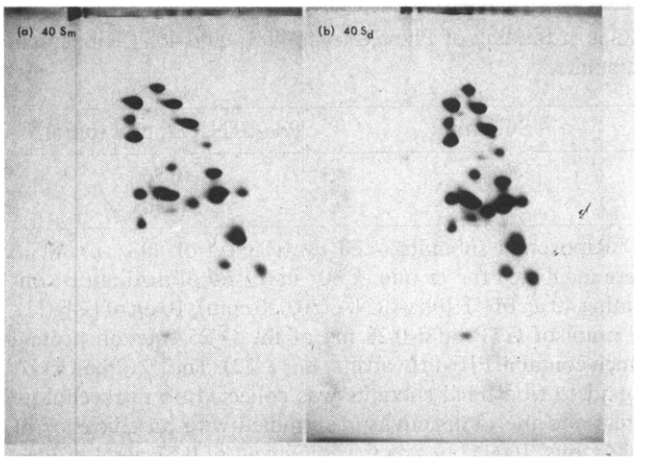

FIGURE 5: Two-dimensional electrophoretograms of the proteins of 40S$_m$ and 40S$_d$ ribosomal subunits. Electrophoresis was in the standard conditions described by Sherton and Wool (1972). The anode was at the left in the first dimension, at the top in the second. The analysis was of 1.2 mg of protein. Note well that an air bubble at the bottom of the gel restricted the migration of the 40S$_d$ proteins in the first dimension. Not all of the proteins of the 40S subunit are separated when standard conditions are used for electrophoresis (Sherton and Wool, 1972); therefore, the presence of those proteins in preparations of the two particles was determined in separate experiments.

40S$_d$ particle must contain less RNA or more protein than the 40S$_m$ particle.

RNA. The RNA was extracted from ribosomal particles with sodium dodecyl sulfate and analyzed on linear sucrose gradients (Figure 4). The 40S$_m$ and 40S$_d$ species contained 18S RNA. Since the 40S$_d$ species had no 28S RNA it cannot be an altered form of the large subunit, nor can it be derived from mitochondrial ribosomes.

Protein. We undertook to determine if there was a difference in the proteins of 40S$_m$ and 40S$_d$ ribosomal subunits. The small subparticle of rat liver ribosomes has 30 different proteins; all 30 can be displayed as single spots when separated by two-dimensional polyacrylamide gel electrophoresis (Sherton and Wool, 1972). No difference was detected in the proteins contained in 40S$_m$ and 40S$_d$ particles (Figure 5). The two particles had all the proteins ordinarily seen in unfractionated preparations of the small ribosomal subunit. Moreover, when a number of gels were compared there was no consistent difference in the amounts of individual proteins as judged from the intensity of the staining of the spots.

Electron Microscopy. The results of the chemical analyses made it very likely that the 40S$_d$ particles were dimers of small ribosomal subunits. We sought to check the assumption by examining preparations of the particles with an electron microscope. Dimers had been observed before in electron micrographs of 40S subunits (Nonomura *et al.*, 1971). Unfixed preparations of 40S$_m$ and 40S$_d$ particles were examined after negative staining with uranyl acetate. (The electron microscopy was done for us by Drs. G. Witman and H. Swift of the University of Chicago.) Preparations of 40S$_m$ particles contained only the monomeric form of the subparticle; preparations of 40S$_d$ subunits contained predominantly dimers (Figure 6). We conclude that the particles with a sedimentation coefficient of 55 (40S$_d$) are dimers of the small subunit.

Low Molecular Weight RNA. The greater buoyant density of the 40S$_m$ particle suggested that it had more RNA or less protein than the 40S$_d$ species. Since we had not been able to find a difference in the proteins of the particles we considered the possibility that they were distinguished by their content of some minor RNA. We had found that preparations of unfractionated 40S ribosomal subunits contained low molecular weight RNA (presumably deacylated tRNA) that would accept amino acids (Wettenhall and Wool, 1973[2]). We undertook then to isolate low molecular weight RNA from 40S$_m$

TABLE IV: Buoyant Density of 40S$_m$ and 40S$_d$ Particles.

Particle	Buoyant Density (g/cm^3)[a]
40S$_m$	1.522 ± 0.002
40S$_d$	1.514 ± 0.003

[a] The values are the mean ± the standard deviation for six determinations (three on each of two separate preparations); the difference is significant ($P < 0.025$).

TABLE III: Translation of EMCV RNA.

Expt	Phenylalanine Incorpd (pmol)[a] 40S$_m$	40S$_d$
1	3.05	3.40
2	2.81	3.39
3	1.54	2.16

[a] Ribosomal subunits (1.27 μg (rRNA) of 40S$_m$ or 40S$_d$ and 5.35 μg (rRNA) of 60S) were incubated for 45 (expt 1 and 2) or 30 min (expt 3) at 37° in 0.05 ml of medium E containing 5 nmol of GTP, 50 nmol of ATP, 90 μg of creatine phosphate, 10 μg of creatine phosphate kinase, 0.25 nmol of [^{3}H]phenylalanine (5 Ci/mmol), 2.5 mmol of each of 19 nonradioactive amino acids, 0.375 mg of ascites cytosol G-25 fraction protein, and 6.6 μg of EMCV RNA. Each 1000 cpm in protein was the equivalent of the incorporation of 0.57 pmol of phenylalanine.

[2] Wettenhall, R. E. H., and Wool, I. G. (1973), unpublished observations.

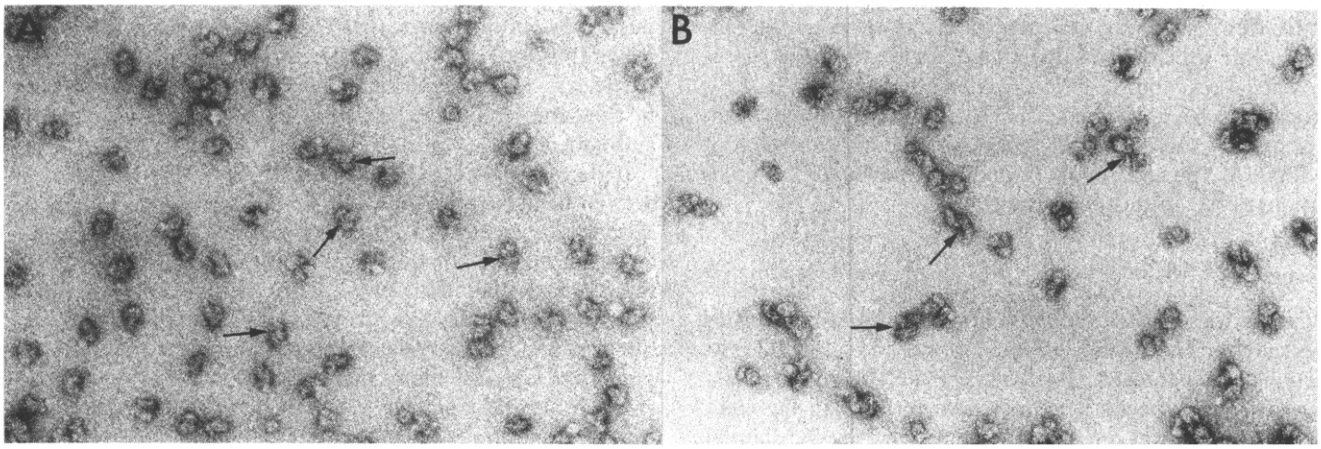

FIGURE 6. Electron micrographs of preparations of $40S_m$ and $40S_d$ negatively stained with uranyl acetate. (A) $40S_m$ (× 150,000); the particles have the elongated profile and transverse dense line (arrows) typical of small ribosomal subunits. (B) $40S_d$ (× 150,000); most of the particles are dimers (arrows) of small ribosomal subunits.

TABLE V: Low Molecular Weight RNA of $40S_m$ and $40S_d$ Particles.

Expt	Low Mol Wt RNA[a] (mol of Deacylated tRNA/mol of Small Ribosomal Subunit) $40S_m$	$40S_d$
1	0.44	0.07
2	0.55	0.05

[a] RNA was extracted from 40S particles with phenol and the ribosomal RNA precipitated with 1 M NaCl. The amount of low molecular weight RNA (deacylated tRNA) was determined from the capacity of the RNA to accept amino acids in the reaction catalyzed by aminoacyl-tRNA synthetases. Comparison was with a standard curve for the aminoacylation of known amounts of rat liver tRNA; in expt 1, each microgram of liver tRNA accepted 2203 cpm of [^{3}H]amino acid; in expt 2, the value was 1738. We have made no correction here for the efficiency of the extraction of RNA which is about 55% (see Methods). For the calculation of the results (moles of deacylated tRNA per mole of small ribosomal subunit) we have assumed the average mol wt of tRNA to be 2.5×10^4 and of 18S RNA to be 6.5×10^5.

and $40S_d$ particles and to compare the amount present in the two species by assessing the capacity of the RNA to accept amino acids (Table V). Both particles contained low molecular weight RNA that accepted amino acids in the reaction catalyzed by aminoacyl-tRNA synthetases; however, in two experiments $40S_m$ subunits had 6.3 and 11 times more of the RNA than $40S_d$ particles. The difference in the amount of low molecular weight RNA bound to the particles could account for the difference in their buoyant density.

It seems reasonable to assume that the low molecular weight RNA is deacylated tRNA. The RNA had a sedimentation coefficient in sucrose gradients of approximately 4 (Figure 7), and it coelectrophoresed in polyacrylamide gels with tRNA (Nakaya and Wool, 1973[3]). The RNA accepts ^{3}H-labeled amino acids in the reaction catalyzed by aminoacyl-tRNA synthetases and the radioactivity cosediments with 4S RNA (Figure 7).

We calculated the proportion of the two species of small ribosomal subparticles that had bound deacylated tRNA. The calculation required that we assume that a comparison of the capacity of a standard preparation of liver tRNA and of low molecular weight RNA extracted from ribosomal subparticles to accept amino acids gave a reliable estimate of total deacylated tRNA in the latter. By that criterion each mole of $40S_m$ had about 0.5 mol of deacylated tRNA (Table V). If we now

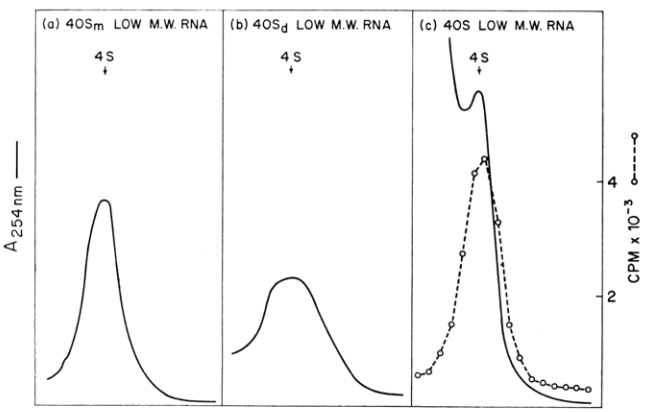

FIGURE 7: Sedimentation of low molecular weight RNA extracted from the 40S ribosomal subunit. Low molecular weight RNA was extracted from 40S particles with phenol and 1 M NaCl. In (a) 6.75 μg of low molecular weight RNA was obtained from 134 μg (rRNA) of the $40S_m$ subunit; in (b) 6.75 μg of low molecular weight RNA was obtained from 235 μg (rRNA) of the $40S_d$ subunit. The low molecular weight RNA (6.75 μg in each case) was analyzed on 5–20% sucrose gradients in 50 mM potassium acetate (pH 6) and centrifuged at 50,000 rpm for 20 hr at 4° in a Spinco SW-65 rotor (a and b). Note that in b the peak is broad and may therefore contain species other than tRNA. In c approximately 35 μg of low molecular weight RNA extracted from unfractionated 40S ribosomal subunits was aminoacylated with a mixture of ^{3}H-labeled amino acids. The low molecular weight RNA was reextracted with phenol and 1 M NaCl and analyzed on a sucrose gradient as in a and b. The material at the top of the gradient is an unknown component of the aminoacylation reaction. The radioactivity was determined by collecting 0.2-ml fractions in glass counting vials: 0.8 ml of water and 10 ml of Triton X-100–toluene scintillation fluid (Patterson and Greene, 1965) were added; the radioactivity was determined in a liquid scintillation counter with an efficiency of 18%.

[3] Nakaya, K., and Wool, I. G. (1973), unpublished observation.

assume that the efficiency of the extraction of deacylated tRNA from ribosomal subparticles is the same as the efficiency with which deacylated tRNA added to 18S RNA is recovered (*i.e.*, about 55%; *cf.* Methods), then each $40S_m$ particle must have a molecule of deacylated tRNA bound to it, whereas only 10–15% of $40S_d$ particles have attached deacylated tRNA. The latter might be due to contamination of preparations of $40S_d$ with $40S_m$ particles.

Discussion

Preparations of small subunits of rat liver ribosomes contain physically and functionally distinct forms of the subparticles: a fraction of the population forms dimers ($40S_d$) in 80 mM potassium. The $40S_d$ species is clearly more active than the $40S_m$ particle. The crucial question is whether the one substantial difference between the two particles—the amount of tightly bound deacylated-tRNA—is sufficient to account for the difference in their physical properties and in their function. The simplest explanation of our findings is that a fraction of the population of small ribosomal subunits are left with deacylated tRNA bound to them, that the deacylated tRNA prevents dimerization of the subunits—perhaps by blocking the site at which they interact or by inducing a conformational change unfavorable for dimerization—and, finally, that particles having bound deacylated tRNA are less active, perhaps because the aminoacyl-tRNA acceptor site is blocked. If that explanation is correct, then the different states of small ribosomal subunits are merely a reflection of our preparative procedures and not likely to be of physiological significance—of importance to scientists but not science. The one reservation we have with that interpretation comes from the observation that addition of deacylated tRNA to $40S_d$ particles does not change their sedimentation, *i.e.*, does not convert dimers to monomers (Nakaya *et al.*, 1973[4]). A decisive test would be to prepare 40S subunits completely free of deacylated tRNA and determine if any of the particles sediment as monomers. However, we have not been able to make the test because we have not yet succeeded in devising procedures for preparing subunits without any tRNA bound to them.

Treatment of eukaryotic ribosomes with puromycin and high concentrations of potassium discharges nascent peptide and much of the tRNA moiety of peptidyl-tRNA (Lawford, 1969; Stirewalt *et al.*, 1971; Blobel and Sabatini, 1971). Some of the tRNA which is not released (Blobel, 1971; Blobel and Sabatini, 1971) could remain on the 40S subunits. If nascent peptide is the source of tRNA on 40S monomers then the proportion of those particles should be directly related to the number of ribosomes engaged in protein synthesis. However, subunits prepared from ribosomes from the muscle of normal and of diabetic animals contain similar proportions of $40S_m$ particles (Wettenhall and Wool[2]); yet a far greater number of normal ribosomes are engaged in the synthesis of protein (Wool and Kurihara, 1967) and hence a greater percentage carry peptidyl-tRNA (Wool and Kurihara, 1967; Castles *et al.*, 1971). Thus, the correlation between the number of ribosomes engaged in protein synthesis and the proportion of $40S_m$ particles derived from the ribosomes is not good.

When subunits are prepared from ribosomes obtained from polysomes by "run-off" (*i.e.*, incubation in conditions where the ribosomes are released from mRNA after completion of nascent chains) the portion of $40S_d$ and $40S_m$ particles is just the same as when the subunits are made from ribosome monomers or by puromycin treatment of the entire ribosome preparation (Nakaya and Wool[3]). Therefore, the formation of $40S_m$ particles is not to be attributed to puromycin since they occur in the same amount when subunits are made without the antibiotic.

If the deacylated tRNA associated with $40S_m$ particles is derived from peptidyl-tRNA it should be bound to a codon specific site on mRNA. We do not know if $40S_m$ particles contain fragments of mRNA, and if they do whether those fragments affect their physical properties and function. Rapidly labeled RNA with a DNA-like base composition (presumed to be mRNA) has been found in preparations of small ribosomal subunits (Mullock *et al.*, 1971; Hamilton *et al.*, 1971), although the mRNA may in fact be associated with cosedimenting nuclear particles (Mullock, *et al.*, 1971).

If we accept for the moment (what is by no means certain) that tRNA on $40S_m$ particles is responsible for their impaired function, then it is most likely that the tRNA interferes with binding of aminoacyl-tRNA. (We assume a single tRNA binding site on the 40S subunit and thereby avoid the complications that arise from consideration of whether deacylated tRNA is in the donor or acceptor site.) If that proposition is correct we would predict there would be no binding of aminoacyl-tRNA to the $40S_m$ subunit, for we calculate that each of the particles has a molecule of tRNA. However, 40S monomers do bind aminoacyl-tRNA, albeit less than half as much as 40S dimers. Of course, our calculation of the number of $40S_m$ subunits that contain tRNA, which was based on assumptions which could not be independently verified, may have been in error. But if the calculation is incorrect and not all $40S_m$ particles have tRNA then we cannot attribute the failure of the particles to dimerize to tRNA. At any rate, the results mitigate against attributing the difference in properties of $40S_m$ and $40S_d$ particles entirely to bound tRNA.

One might suppose that only 40S monomers lacking deacylated tRNA would actually bind aminoacyl-tRNA and hence show activity. Approximately 20% of the particles in preparations of $40S_d$ subunits bind Phe-tRNA; only 8% of $40S_m$ subunits will do so. If the supposition is correct then 8% of the particles in preparations of $40S_m$ subunits should lack deacylated tRNA—our measurements do not exclude that possibility. However, it would be necessary also for some 20% of the particles in preparations of $40S_d$ to exist as monomers free of deacylated tRNA and that is less likely, but once again cannot be ruled out with certainty.

It is possible that $40S_m$ and $40S_d$ particles are separate conformational states of the small ribosomal subunit, that one of the conformational states ($40S_m$) is either induced by deacylated tRNA or causes deacylated tRNA to remain tightly bound to the $40S_m$ particle, and, finally, that the less favorable conformation ($40S_m$) decreases the efficiency of binding of aminoacyl-tRNA. There is circumstantial evidence for a difference in the conformation of monomers and dimers of the small subunit of *Escherichia coli* ribosomes (Zamir *et al.*, 1971), although it is not certain whether the active species is the monomer (Zamir *et al.*, 1971) or the dimer (Pestka and Nirenberg, 1966). Nonetheless, the important observation is that the conformation can be changed and the particles activated by repletion of magnesium ions or potassium or both in a temperature-dependent reaction (Zamir *et al.*, 1969, 1971; Kikuchi and Monier, 1970). If ribosomal subunits can take altered conformations the several forms are likely to differ in function; alterations in conformation might provide an explanation for the role of the ribosome in the

[4] Nakaya, K., Wettenhall, R. E. H., and Wool, I. G. (1973), unpublished observation.

partial reactions of protein synthesis and might provide a means for control of ribosome activity.

References

Aviv, H., Boime, I., and Leder, P. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2303.

Bellet, A. J. D., and Burness, A. T. H. (1963), *J. Gen. Microbiol. 30*, 131.

Blobel, G. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1881.

Blobel, G., and Sabatini, D. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 390.

Castles, J. J., Rolleston, F. S., and Wool, I. G. (1971), *J. Biol. Chem. 246*, 1799.

Ceccarini, C., Andronico, F., and Campo, M. S. (1970), *Biochim. Biophys. Acta 217*, 212.

Dube, S. K., Marcker, K. A., Clark, B. F. C., and Cory, S. (1969), *Eur. J. Biochem. 8*, 244.

Eikenberry, E. F., Bickle, T. A., Traut, R. R., and Price, C. A. (1970), *Eur. J. Biochem. 12*, 113.

Enger, M. D., and Walters, R. A. (1970), *Biochemistry 9*, 3551.

Gilbert, W. (1963), *J. Mol. Biol. 6*, 389.

Hamilton, M. G., Pavlovec, A., and Petermann, M. L. (1971), *Biochemistry 10*, 3424.

Hardy, S. J. S., Kurland, C. G., Voynow, P., and Mora, G. (1969), *Biochemistry 8*, 2897.

Henshaw, E. C., and Loebenstein, J. (1970), *Biochim. Biophys. Acta 199*, 405.

Ifft, J. B., Voet, D. M., and Vinograd, J. (1961), *J. Phys. Chem. 63*, 1138.

Igarashi, K., and Kaji, A. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 498.

Infante, A. A., and Krauss, M. (1971), *Biochim. Biophys. Acta 246*, 81.

Kaltschmidt, E., and Wittmann, H.-G. (1970), *Anal. Biochem. 36*, 401.

Kaulenas, M. S. (1971), *Anal. Biochem. 41*, 126.

Kerr, I. M., Cohen, N., and Work, T. S. (1966), *Biochem. J. 98*, 826.

Kikuchi, A., and Monier, R. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 11*, 157.

Lawford, G. R. (1969), *Biochem. Biophys. Res. Commun. 37*, 143.

Leader, D. P., Klein-Bremhaar, H., Wool, I. G., and Fox, A. (1972), *Biochem. Biophys. Res. Commun. 46*, 215.

Leader, D. P., Wool, I. G., and Castles, J. J. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 523.

Martin, T. E., and Wool, I. G. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 569.

Martin, T. E., and Wool, I. G. (1969), *J. Mol. Biol. 43*, 151.

Martin, T. E., Wool, I. G., and Castles, J. J. (1971), *Methods Enzymol. 20*, 417.

Mathews, M. B., and Korner, A. (1970), *Eur. J. Biochem. 17*, 328.

Mosteller, R. D., Culp, W. J., and Hardesty, B. (1968), *J. Biol. Chem. 243*, 6343.

Mullock, B. M., Hinton, R. H., Dobrota, M., Froomberg, D., and Reid, E. (1971), *Eur. J. Biochem. 18*, 485.

Nonomura, Y., Blobel, G., and Sabatini, D. (1971), *J. Mol. Biol. 60*, 303.

Patterson, H. S., and Greene, R. C. (1965), *Anal. Biochem. 37*, 854.

Pestka, S., and Nirenberg, M. (1966), *J. Mol. Biol. 21*, 145.

Petermann, M. L., and Pavlovec, A. (1971), *Biochemistry 10*, 2770.

Shafritz, D. A., and Anderson, W. F. (1970), *J. Biol. Chem. 245*, 5553.

Sherton, C. C., and Wool, I. G. (1972), *J. Biol. Chem. 247*, 4460.

Spirin, A. S., Belitsina, N. V., and Lerman, M. I. (1965), *J. Mol. Biol. 14*, 611.

Stirewalt, W. S., Castles, J. J., and Wool, I. G. (1971), *Biochemistry 10*, 1594.

Tashiro, Y., and Morimoto, T. (1966), *Biochim. Biophys. Acta 123*, 523.

Terao, K., and Ogata, K. (1970), *Biochem. Biophys. Res. Commun. 38*, 80.

von Ehrenstein, G., and Lipmann, F. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 941.

Wool, I. G., and Cavicchi, P. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 991.

Wool, I. G., and Cavicchi, P. (1967), *Biochemistry 6*, 1231.

Wool, I. G., and Kurihara, K. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 2401.

Zamir, A., Miskin, R., and Elson, D. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 3*, 85.

Zamir, A., Miskin, R., and Elson, D. (1971), *J. Mol. Biol. 60*, 347.