

Dissociation of N₂ Gas-induced Monomeric Ribosomes and Functioning of the Derived Subunits in Protein Synthesis in Pea¹

Received for publication March 9, 1971

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ABSTRACT

The dissociation of N₂ gas-induced monomeric ribosomes from the pea root was studied by varying the concentration of KCl (or NH₄Cl) and MgCl₂ in the presence of dithiothreitol. These monoribosomes were shown to dissociate completely into subunits at 0.5 M KCl or NH₄Cl in the presence of 5 mM MgCl₂. The 40S subunits were more susceptible to structural change in KCl than were the 60S subunits. On the other hand, the 60S subunits appeared to be more labile to NH₄Cl.

The activity of the subunits relative to aminoacyl-tRNA binding and peptide bond formation was investigated using subunits derived from 0.5 M KCl (or NH₄Cl) in the absence and presence of 5 mM MgCl₂. The 40S subunits were active in aminoacyl-tRNA binding only when dissociated in the presence of MgCl₂. The 40S and 60S subunits combined in the presence of poly U were active in incorporation of ¹⁴C-phenylalanine from ¹⁴C-phenylalanyl-tRNA only when dissociation was achieved in the presence of 5 mM MgCl₂. The KCl-dissociated subunits were much more active in protein synthesis than NH₄Cl-dissociated subunits.

Bacterial ribosomes have been readily dissociated into active subunits by lowering the magnesium ion concentration (7, 23, 25). With mammalian ribosomes (polyribosomes preparation), incubation in a high concentration of monovalent cation in the absence of MgCl₂ has only resulted in a decrease in the proportion of polyribosomes (3), while complete dissociation into subunits required a high pH or a chelating agent such as EDTA (6). However, subunits derived in this way were not active in *in vitro* protein synthesis (3, 6). The reticulocyte ribosomal subunits obtained by KCl shocking in the presence of dithiothreitol were active in binding of aminoacyl-tRNA to the small subunits and also active in protein synthesis (8). Martin *et al.* (15) have also demonstrated that monomeric mammalian ribosomes derived from puromycin-treated polyribosomes could be dissociated into subunits in a high concentration of KCl in the presence of magnesium ion. These salt-dissociated subunits were active in protein synthesis.

Dickman and Bruenger (5) have purified dog pancreatic ribosomal subunits by adding ribosomes to a DEAE-cellulose column followed by elution with a KCl gradient. This technique effectively dissociated and separated ribosomes into subunits which were active in *in vitro* protein synthesis.

Plant ribosomes have been previously dissociated into subunits in a phosphate buffer in the absence of magnesium (3, 20, 26). But at present there is no evidence that plant ribosomes can be dissociated into subunits which are active in aminoacyl-tRNA binding and in amino acid incorporation into protein.

Numerous workers (11, 13, 18, 21, 22) have reported the use of NH₄Cl to wash ribosomes free of endogenous enzyme activity in studies of GTP-dependent enzymatic binding of aminoacyl-tRNA to ribosomes. In this paper we report the dissociation behavior of pea ribosomes using different concentrations of NH₄Cl and KCl in the presence or absence of magnesium ion. Conditions are also reported for the dissociation of ribosomes into subunits which are active in *in vitro* amino acid incorporation.

MATERIALS AND METHODS

Preparation of Monomeric Ribosomes. Pea seeds were germinated in rolls of moist paper (10). Three-day-old seedlings were immersed in distilled H₂O and bubbled with N₂ gas for 1 hr at room temperature in order to dissociate polyribosomes into monomeric ribosomes (12).

Apical (tip 5-mm sections of pea roots of 600-800 seedlings) were cut into ice-cold 0.25 M sucrose solution containing tris buffer (50 mM tris, pH 7.5, 20 mM KCl, and 5 mM MgCl₂). Homogenization was accomplished with a Willems Polytron PT 20 st. at speed 8 for 8 sec. The homogenate was filtered through Miracloth, and the filtrate was centrifuged at 13,500g for 15 min. Ribosomes were pelleted from the supernatant solution by layering over 3 ml of sucrose solution (1.0 M sucrose containing tris buffer) followed by centrifugation at 229,400g for 85 min. All steps were carried out at 0 to 4 C.

Dissociation of Monomeric Ribosomes and Preparation of Subunits. Ribosomes were suspended in 50 mM tris buffer containing various concentrations of NH₄Cl or KCl with or without MgCl₂ as indicated in the figure legends. Ribosomal subunits were separated on 15 to 30% linear sucrose gradients containing 1 mM dithiothreitol in addition to the same concentrations of salts used for ribosomal suspension. Centrifugation was at 39,000 rpm in a Spinco SW 41 rotor for 5 hr at 3 C. The distribution of ribosomal subunits in the sucrose gradients was monitored with a continuous recording ISCO model D density gradient fractionator. For preparative purposes ribosomal subunits were first separated on 15 to 30% linear sucrose gradients in a Spinco SW 27 rotor at 25,000 rpm for 15 hr. Fractions containing the respective 40S and 60S subunits were pooled and dialyzed at 4 C overnight against tris buffer containing mM dithiothreitol. The dialyzed subunit fractions were pelleted in a Spinco T 65 rotor at 229,400g for 4 hr. The purity of each preparation was checked by acrylam-

¹This research was supported by Atomic Energy Commission Contract AT(38-1)-643 to Joe L. Key.

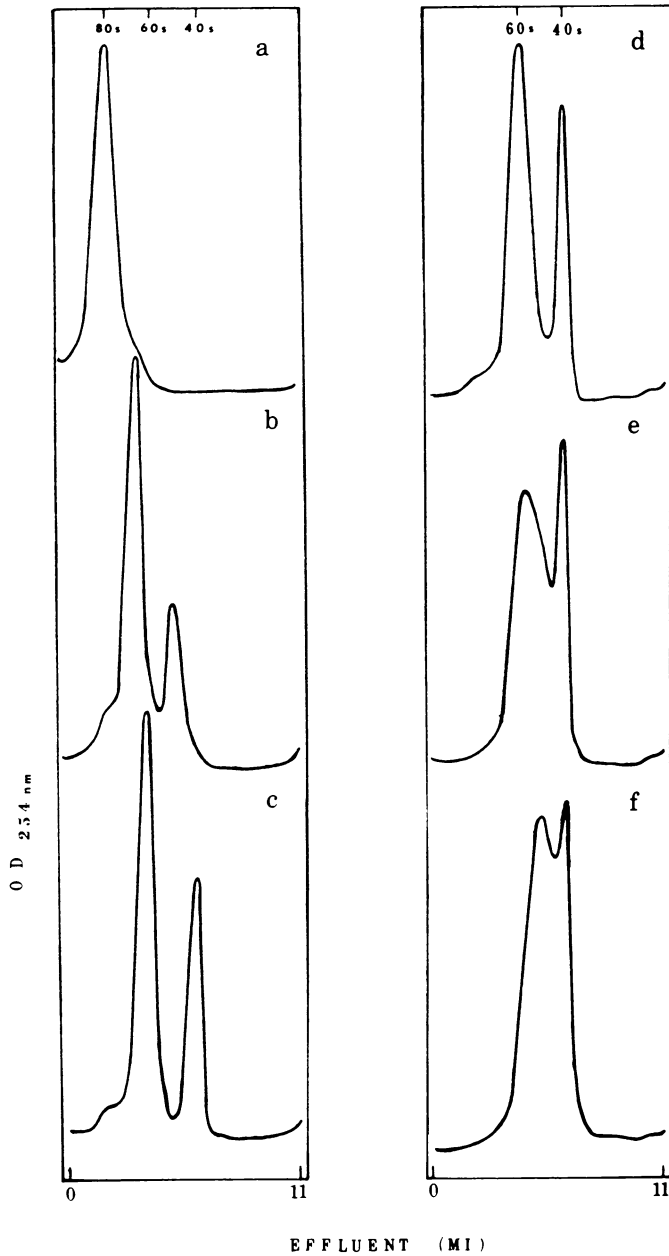


FIG. 1. Sucrose gradient profile of ribosomes dissociated by NH_4Cl in the presence of 5 mM MgCl_2 . a: Control (minus NH_4Cl); b: 0.1 M NH_4Cl ; c: 0.3 M NH_4Cl ; d: 0.5 M NH_4Cl ; e: 0.8 M NH_4Cl ; f: 1.0 M NH_4Cl .

ide gel electrophoresis of the RNA extracted from the subunits and also by recentrifugation of the subunits separately on sucrose gradients containing tris buffer.

Preparation of Aminoacyl-tRNA Binding and Transfer Enzymes. Aminoacyl-tRNA binding and transfer enzymes were prepared by $(\text{NH}_4)_2\text{SO}_4$ fractionation (45–65%) of the post-ribosomal supernatant followed by gel filtration as described previously (13).

Preparation of ^{14}C -Phenylalanyl-tRNA. Preparation of pea tRNA and acylation of ^{14}C -phenylalanine (450 mc/mole) to pea tRNA were carried out according to the methods of Vanderhoef *et al.* (27).

Aminoacyl-tRNA Binding and Transfer Assays. The binding assay was carried out in 0.5 ml of reaction mixture containing 50 mM HEPES, pH 7.5; 80 mM KCl; 80 mM NH_4Cl ;

10 mM MgCl_2 ; 0.3 mM GTP; 40 to 50 μg of enzyme fraction; 50 μg of poly U; 2.0 A_{260} of ribosomes; and the indicated amount of ^{14}C -phenylalanyl-tRNA (36,280 cpm/1.0 A_{260} tRNA).

Incubation was for 10 min at 0 C, after which time the reaction was terminated by dilution followed by filtration on a Millipore membrane (HA 0.45 μ) according to the method of Nirenberg and Leder (17). The membrane was then dried and counted in a liquid scintillation spectrometer. For assay of amino acid transfer (peptide bond formation) the reaction mixture was incubated for 20 min at 37 C, after which 10% trichloroacetic acid was added to a final concentration of 5%. The acidified reaction mixture was then heated at 90 C for 15 min. Hot trichloroacetic acid-insoluble radioactivity retained on Whatman GFA glass fiber discs was counted in a liquid scintillation spectrometer as a measure of peptide formation.

The details of GTP-dependent enzymatic binding of aminoacyl-tRNA to ribosomes and amino acid transfer are in preparation for publication.

RESULTS

Dissociation of Ribosomes with NH_4Cl . The dissociation behavior of monomeric ribosomes in response to different concentrations of NH_4Cl at 5 mM MgCl_2 is shown in Figure 1. Dissociation was considered complete at 0.3 M to 0.5 M NH_4Cl as shown in Figure 1c and 1d, although there still remained a small residual portion of ribosomes which were resistant to dissociation at 0.3 M salt. As the salt concentration was increased above 0.5 M, the 60S subunit sedimented more slowly than at the lower salt concentrations. This effect was most pronounced at 1.0 M, the highest salt concentration tested in the presence of 5 mM MgCl_2 (Fig. 1f). However, the 40S subunit was not affected by NH_4Cl concentrations up to 1.0 M in the presence of 5 mM MgCl_2 .

When 10 mM MgCl_2 was present in the NH_4Cl dissociation medium, somewhat different results were obtained. As shown in Figure 2, at 10 mM MgCl_2 , the NH_4Cl concentration necessary to dissociate the ribosomes was still 0.5 M, as was the case when 5 mM MgCl_2 was present. However, even at NH_4Cl concentrations as high as 1.0 M, sedimentation of the 60S subunit was unaffected by the high salt (Fig. 2b, 2c, and 2d). At 1.5 M and 2.0 M NH_4Cl (10 mM MgCl_2) both the 60S and 40S subunits sedimented at a slower rate than was observed at lower salt concentrations (Fig. 2e and 2f). It is not clear whether these salt effects on sedimentation rate are the result of conformational changes of the subunits or whether they are a result of the loss of protein from the subunits (or both).

Dissociation with KCl. In the presence of 5 mM MgCl_2 , dissociation of monomeric ribosomes was complete at 0.5 M KCl as was observed for NH_4Cl . At 0.8 M KCl (5 mM MgCl_2), both the 60S and 40S subunits sedimented at a slower rate (Fig. 3f). In addition, this decreased rate appeared to be accomplished in a stepwise fashion as evidenced by the shoulders on both subunit peaks (Fig. 3g and 3h). At 10 mM MgCl_2 , this stepwise decrease in sedimentation rate with increasing KCl concentration was more pronounced (Fig. 4d, 4e and 4f). With 10 mM MgCl_2 , dissociation of ribosomes into subunits was observed at 0.5 M KCl, as was the case with 5 mM MgCl_2 . However, at the high MgCl_2 concentration the decrease in sedimentation rate of the 60S subunit with the increasing KCl concentration occurs in at least two steps, as shown in Figure 4e. This is in contrast to the 40S subunit where the decrease in sedimentation occurred as a single step (Fig. 4d). At 10 mM MgCl_2 it was also observed that the decrease in sedimentation rate of the 40S subunit occurred at a lower KCl concentration than was the case for the 60S subunit. From the

results of our experiments it was not certain whether the latter occurred at 5 mM MgCl₂; however, the decrease in sedimentation rate of the 40S subunit seemed to occur at a lower KCl concentration than was the case for the 60S subunit (see Figs. 3g and 4d).

In the absence of MgCl₂, dissociation of ribosomes into subunits occurred at concentrations as low as 50 mM KCl (Fig. 5a). However, it is interesting to note that the decrease in sedimentation rate occurred at 0.5 M (Fig. 5d) as compared to 0.8 M KCl with 5 mM MgCl₂ (Fig. 3g) and 1.0 M KCl at 10 mM MgCl₂ (Fig. 4d). In addition there was no indication that this decrease in sedimentation rate occurred in a stepwise fashion as was observed with 10 mM MgCl₂. Again, the reason for the decrease cannot be determined from our experiments. However, with no MgCl₂ present a peak of ultraviolet-absorbing material was observed in the low molecular weight region

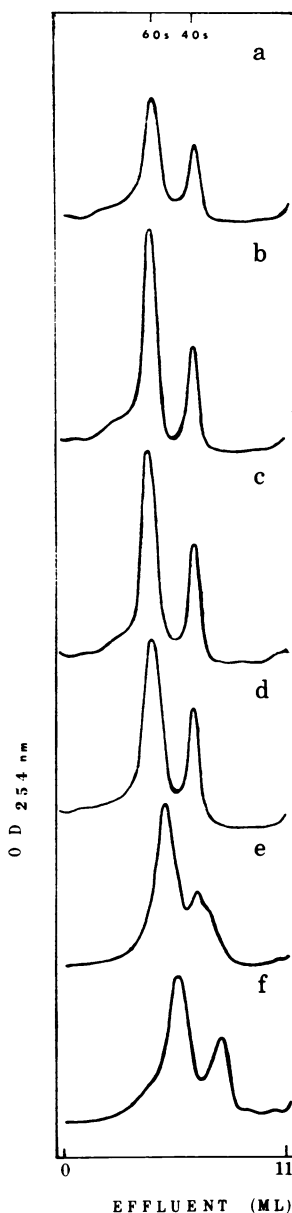


FIG. 2. Sucrose gradient profile of ribosomes dissociated by NH₄Cl in the presence of 10 mM MgCl₂. a: Control (0.5 M NH₄Cl and 5 mM MgCl₂); b: 0.5 M NH₄Cl; c: 0.8 M NH₄Cl; d: 1.0 M NH₄Cl; e: 1.5 M NH₄Cl; f: 2.0 M NH₄Cl.

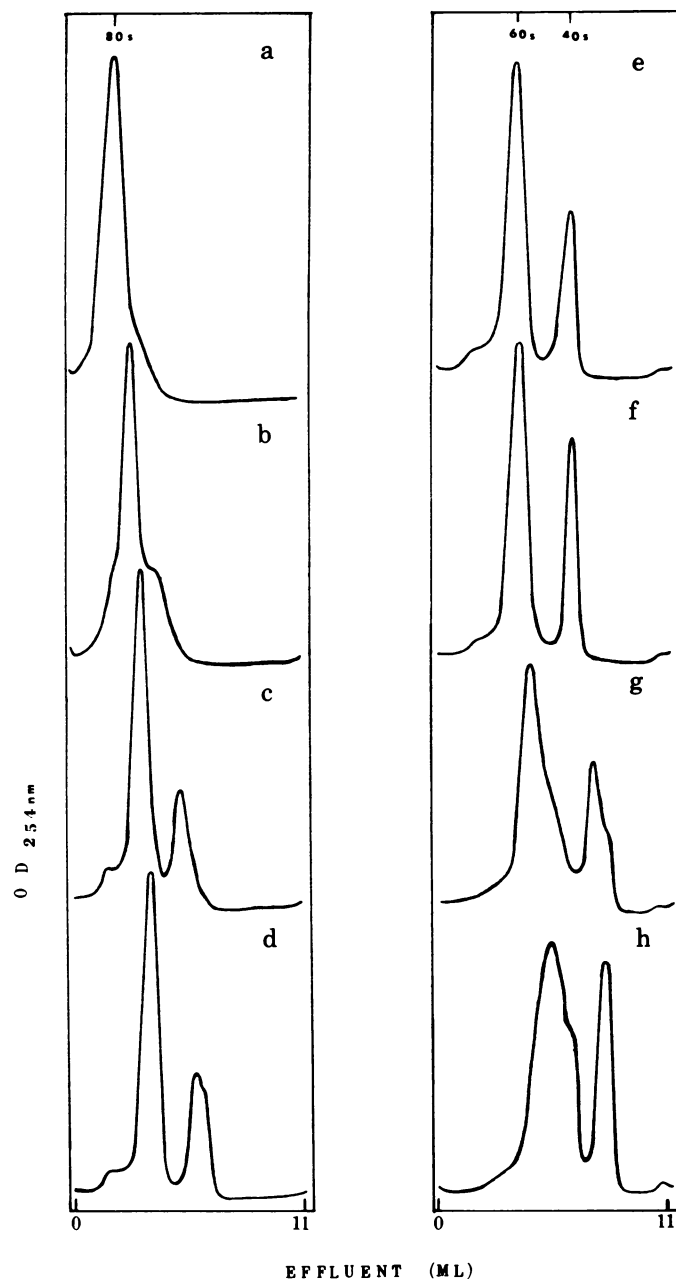


FIG. 3. Sucrose gradient profile of ribosomes dissociated by KCl in the presence of 5 mM MgCl₂. a: Control (minus KCl); b: 0.1 M KCl; c: 0.2 M KCl; d: 0.3 M KCl; e: 0.4 M KCl; f: 0.5 M KCl; g: 0.8 M KCl; h: 1.0 M KCl.

of the gradient. It is possible that this peak represents protein which was dissociated from the subunits, resulting in a decrease in sedimentation rate.

Aminoacyl-tRNA Binding by 40S Subunits. The 40S subunits obtained by dissociation with either KCl or NH₄Cl in the presence of 5 mM MgCl₂ possessed phenylalanyl-tRNA binding activity (Table I). This binding was poly U-specific and enzyme-dependent, although the subunits retained some "non-enzymatic" binding activity. This latter binding activity may, however, be the result of residual enzyme still associated with the 40S subunit preparation. Enzymatic binding to the subunit was not substantially inhibited by GMPPCP,² a GTP

² Abbreviation: GMPPCP: guanylyl-5'-methylene diphosphonate.

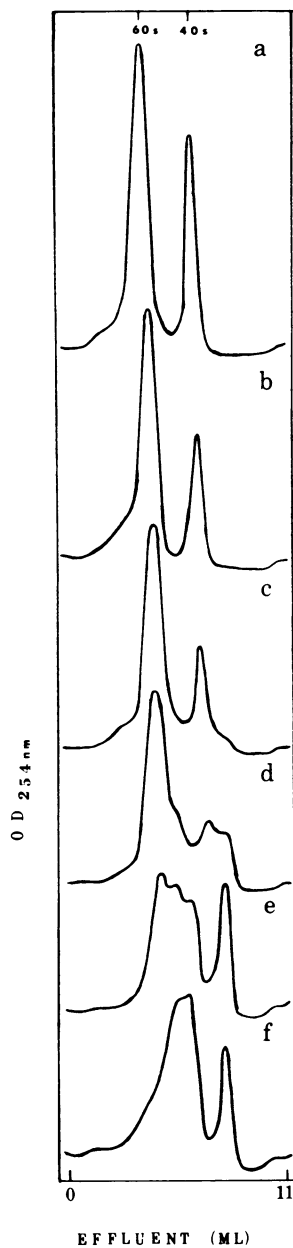


FIG. 4. Sucrose gradient profile of ribosomes dissociated by KCl in the presence of 10 mM $MgCl_2$. a: Control (0.5 M KCl and 5 mM $MgCl_2$); b: 0.5 M KCl; c: 0.8 M KCl; d: 1.0 M KCl; e: 1.5 M KCl; f: 2.0 M KCl.

analogue. Subunits obtained in the absence of $MgCl_2$ did not bind phenylalanyl-tRNA to the same extent as did those obtained in the presence of $MgCl_2$, and it should be noted that the subunits obtained in the absence of $MgCl_2$ sedimented more slowly than did those obtained in the presence of $MgCl_2$ (Fig. 5d). The decrease in sedimentation rate observed using different conditions of dissociation was accompanied by a loss of aminoacyl-tRNA binding capacity of the subunits.

Although significant enzymatic binding was observed with 60S subunits, the 40S subunits exhibited by far the greater binding activity. It cannot be ascertained whether or not this reflects a contamination of 60S subunits by 40S subunits or if indeed this reflects a separate binding reaction of aminoacyl-tRNA to the 60S subunits. Gel electrophoresis of the RNA obtained from either subunit preparation did not reveal any contamination by the other (data not shown).

Protein Synthetic Activity of Ribosomal Subunits. Protein synthetic activity of ribosomal subunits dissociated under different salt conditions is shown in Table II. Subunits dissociated by KCl in the absence of $MgCl_2$ lacked protein synthetic activity while those dissociated in the presence of $MgCl_2$ were able to incorporate amino acids into protein. Considerably greater activity was observed with KCl-dissociated subunits than with NH_4Cl -dissociated subunits. Protein synthetic activity was poly U- and enzyme-dependent and was inhibited by GMPPCP. Either subunit alone possessed only a fraction of the activity observed for the combined 60S and 40S subunits, with the 60S fraction being the more active of the two subunits when assayed separately. Although the 60S subunit alone had 25% the activity observed for the combined subunits, it was not possible to attribute this activity to contamination of the

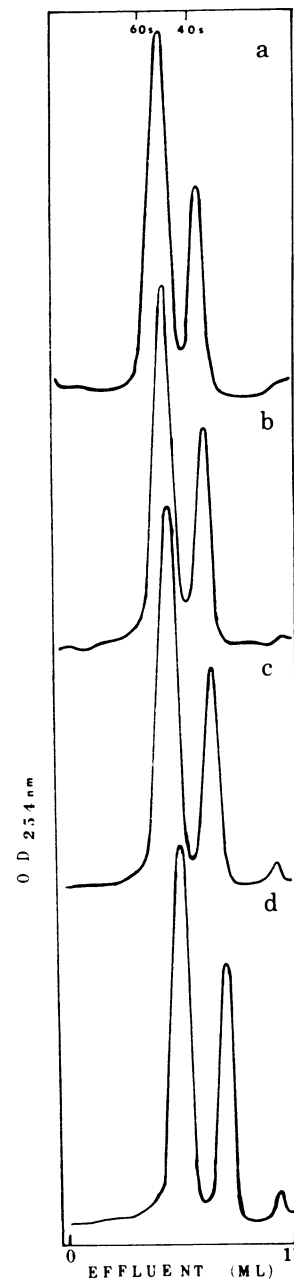


FIG. 5. Sucrose gradient profile of ribosomes dissociated by KCl in the absence of $MgCl_2$. a: 50 mM KCl; b: 0.1 M KCl; c: 0.3 M KCl; d: 0.5 M KCl.

60S subunits with 40S subunits. Gel electrophoresis of RNA extracted from 60S subunits did not reveal the presence of 18S RNA in the preparation. Protein synthetic activity was approximately the same if subunits were dialyzed either separately or together. In addition, it was found that active subunits could be obtained only if 1 mM dithiothreitol was present during dissociation and dialysis.

DISCUSSION

Martin *et al.* (16) demonstrated that complete dissociation of rat muscle monoribosomes could be accomplished in a 0.88 M KCl-10 mM MgCl₂ solution at 28 C. With this method 40S subunits at 4 C formed dimers which cosedimented with 60S subunits on sucrose density gradients, hampering the isolation of purified subunits. With pea monoribosomes, dissociation was accomplished at 4 C with no apparent dimerization of the subunits. The present method proved useful for purification of active 40S and 60S subunits.

With *E. coli* ribosomes, the presence of magnesium ion interferes with release of protein from the ribosomes. In this system, different salts produce different effects (*e.g.*, NaCl detaches large amounts of protein while NH₄Cl removes very little if any) (1, 24). These results were based on studies with whole ribosomes rather than the individual subunits. Here we report the qualitative changes in sedimentation of each ribosomal subunit with different concentrations of KCl or NH₄Cl. In our experiments the 40S subunit proved to be more susceptible to structural change to KCl while, on the other hand, the 60S subunit was more labile to NH₄Cl at the salt concentrations tested. The amount of protein released from the subunits varied markedly with the conditions used, increasing with higher KCl or NH₄Cl concentrations and decreasing with increasing magnesium ion concentration.

Subunits dissociated with either 0.5 M KCl or 0.5 M NH₄Cl in the presence of 5 mM MgCl₂ were active in aminoacyl-tRNA binding (40S subunit, but the NH₄Cl-dissociated subunits (40S + 60S) were much less active than the KCl-derived subunits in peptide bond formation. Dissociation conditions which resulted in loss of binding capacity or over-all protein synthetic activity could not be attributed to a loss of ability of the subunits to reassociate as monomeric ribosomes. In fact, in all cases, dissociation resulted in subunits which were able to recombine into monomeric ribosomes in the presence of tris buffer (data not shown).

Pea 40S ribosomal subunits bind phenylalanyl-tRNA in the presence of poly U, GTP, and enzyme, although there was not an absolute requirement for added enzyme. Relative to the 40S subunit, little or no binding was detected for 60S subunits with phenylalanyl-tRNA. In general the requirements for enzymatic binding of aminoacyl-tRNA to pea 40S subunits are similar to those reported for mammalian 40S subunits (8, 21) and bacterial 30S subunits (14). The low level of binding of aminoacyl-tRNA to the small subparticles observed here in the absence of supernatant enzyme is similar to that which has been reported for both procaryote and eucaryote ribosomes (4, 9, 19). NH₄Cl-dissociated subparticles showed much less activity in peptide bond formation than those dissociated by KCl. Since aminoacyl-tRNA binding activity to 40S subunits was similar for KCl- and NH₄Cl-dissociated particles, it is possible that NH₄Cl might detach some enzymes which are essential for protein synthesis.

It is difficult to explain some "protein synthetic activity" by 60S subunits in the absence of detectable 40S subunits in the presence of added template. One possible explanation is a contamination by 40S subunits which was not detectable by sucrose gradient centrifugation and acrylamide gel electro-

Table I. Binding of ¹⁴C-Phenylalanyl-tRNA to Subunits of Pea Ribosomes

Reaction Mixture	¹⁴ C-Phenylalanyl-tRNA Bound					
	NH ₄ Cl ¹		KCl ¹		KCl, - MgCl ₂ ²	
	- Enzyme	+ Enzyme	- Enzyme	+ Enzyme	- Enzyme	+ Enzyme
	<i>cpm</i>					
40S	100	345	125	275	60	95
60S	45	120	25	85	80	115
40S, -poly U	...	90	...	80
40S, -GTP, + GMPPCP	...	225	...	270
40S + 60S ³	95	435

¹ Ribosomal subunits were obtained by dissociation with 0.5 M NH₄Cl and 0.5 M KCl in the presence of 5 mM MgCl₂, respectively; 14,100 cpm of ¹⁴C-phenylalanyl-tRNA were used per assay.

² Ribosomal subunits were obtained by dissociation with 0.5 M KCl in the absence of MgCl₂; 13,850 cpm of ¹⁴C-phenylalanyl-tRNA were used per assay.

³ 0.7 A₂₆₀ unit of 40S; 1.4 A₂₆₀ units of 60S.

Table II. Polyphenylalanine Synthesis by Combination of Subunits of Pea Ribosomes

Conditions were the same as in Table I.

Reaction Mixture	¹⁴ C-Polyphenylalanine					
	NH ₄ Cl		KCl		KCl, -MgCl ₂	
	- Enzyme	+ Enzyme	- Enzyme	+ Enzyme	- Enzyme	+ Enzyme
	<i>cpm</i>					
40S	70	70	115	265	65	70
60S	65	195	135	2540	55	60
40S + 60S	60	835	135	9600	70	45
60S, -poly U	...	90	...	245
60S, -GTP, + GMPPCP	...	80	...	285
40S + 60S, -poly U	...	75	...	190
40S + 60S, -GTP, + GMPPCP	...	80	...	135

phoresis (the latter method would detect slight cross contamination) of RNA extracted from each of the subparticles.

Previously we demonstrated that the conversion of polyribosomes to monomeric ribosomes under anaerobiosis was due to the completion of read-out of the messenger RNA and release of nascent protein (12). The data in the present report offer further evidence for that hypothesis by the following argument. Ribosomes associated with mRNA and aminoacyl-tRNA are resistant to complete dissociation by salt into subunits (15). In this study subunits were obtained by first treating the seedlings with N₂ gas and then by salt dissociation of the resultant monoribosomes. Presumably these monoribosomes lack mRNA which protects the ribosomes from salt dissociation, a condition expected if the monoribosomes are formed as a result of read-out of the mRNA and release of nascent protein and monoribosomes.

App *et al.* (Plant Physiol. 47: 81, 1971) recently reported the dissociation of ribosomes from rice embryo by 0.5 M KCl-4 mM MgCl₂. The derived subunits were active in poly U-directed incorporation of phenylalanine.

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