Presumably the structures of $tRNA^{Val}$ (*E. coli*) and $tRNA^{Phe}$ (*N. crassa*) are quite similar; otherwise, the heterologous charging reactions that have been observed would not be expected to occur. Consequently, we cannot say to what extent different bases in the vicinity of the aminoacyl bond might affect stability. Since details of tRNA tertiary structure are not yet available, it is unclear which areas of the molecule might be in the vicinity of the -C-C-A end under various conditions. It is clear, however, from our data and that of others that factors affecting tRNA structure change the rates of nonenzymic hydrolysis of aminoacyl-tRNAs. Using various techniques, a number of studies have shown changes in tRNA conformation induced by low levels of magnesium (Eisinger *et al.*, 1970; Zimmerman *et al.*, 1970; Beardsley *et al.*, 1970; Robison and Zimmerman, 1971; Willick and Kay, 1971; Rosenfeld *et al.*, 1970; Römer *et al.*, 1970). The greatest changes appeared to take place between 0 and 10 mM Mg^{2+} (Willick and Kay, 1971). High concentrations of NaCl mimicked to a lesser degree the effects of lower Mg^{2+} concentrations in fluorescence studies with $tRNA^{Phe}$ of beef liver (Zimmerman *et al.*, 1970) and yeast (Robison and Zimmerman, 1971). This is consistent with our results that equivalent effects on rates of hydrolysis of aminoacyl-tRNAs were achieved by Mg^{2+} and KCl when the concentration of the latter was 100–200 times that of the former.

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A 5S Ribonucleic Acid–Protein Complex Extracted from Rat Liver Ribosomes by Formamide†

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ABSTRACT: When rat liver ribosomes were dialyzed against 30 mM KCl–0.2 mM $MgCl_2$–3 mM potassium phosphate, pH 7.3, and formamide was added to a final concentration of 8.9 M, in the cold, the 5S RNA was detached in the form of a nucleoprotein complex (RNP). This complex was isolated by differential centrifugation and by fractionation on G-200 Sephadex, where its elution volume corresponded to a molecular weight of about 80,000. Its protein had a molecular weight of 41,000, measured by gel electrophoresis in sodium dodecyl sulfate. The RNP complex was unstable, being easily dissociated by moderate concentrations of electrolyte. After formaldehyde fixation it had a sedimentation coefficient of 6.8 S; in a cesium chloride gradient it banded at a density of 1.568 g/cm^3, corresponding to an RNA content of 48.5%.

Although the 5S RNA, found in the large subunits of both 70S and 80S ribosomes (Attardi and Amaldi, 1970) appears to be essential for protein-synthetic activity, its function is still unknown. When it is extracted from the ribosome by EDTA treatment a fraction of it is found in a ribonucleoprotein complex (RNP)[1] (Mazelis, 1970; Lebleu *et al.*, 1971; Petermann *et al.*, 1971; Blobel, 1971). With rat liver ribosomes a higher ratio of complex to free RNA is found when the RNP is extracted with formamide (Petermann *et al.*, 1971). This paper describes the extraction of the RNP, its stability, its partial purification, and some of its physical properties.

† From the Sloan-Kettering Institute for Cancer Research, New York, New York 10021. *Received January 12, 1972.* This investigation was supported by Research Grant CA 08748 from the National Cancer Institute, U. S. Public Health Service, and by Atomic Energy Commission Contract AT (30-1)-910. A portion of this work was presented at the American Society of Biological Chemists, San Francisco, Calif., June 16, 1971.

[1] Abbreviation used is: RNP, the 5S RNA–protein complex.

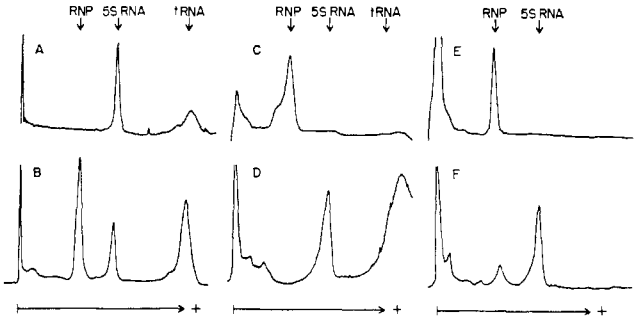

FIGURE 1: Densitometer tracings of polyacrylamide gel electrophoreses of formamide extracts. A, marker RNAs; B, C, and D, extract of whole ribosomes; E and F, extract of large subunits. A, B, D, and F, RNA stain; C and E, protein stain. D, treated with sodium dodecyl sulfate before electrophoresis; some of the color in the tRNA region represents sodium dodecyl sulfate. The sharp peaks at the left of the tracings represent ribosomal subunits or RNAs that remained in the sample slots.

Methods

Adult male rats were pretreated with phenobarbital to decrease the concentration of RNase in the liver, and ribosomes were isolated as described by Petermann and Pavlovec (1971), except that only 0.5% deoxycholate was used. They were stored at −20° in 2% sucrose–1 mM dithiothreitol–0.2 mM $MgCl_2$–1 mM potassium phosphate, pH 7.3. To extract the RNP 12 ml of solution, containing 80 mg of ribosomes, was used. KCl was added to a final concentration of 100 mM, and the solution was dialyzed in a Zeineh dialyzer (Biomed Instruments Inc., Chicago, Ill.) for 2.5 hr at 5° against buffer containing 30 mM KCl–0.1 mM $MgCl_2$–3 mM potassium phosphate, pH 7.3. Formamide was adjusted with 0.2 N HCl until a sample, diluted with dialysis buffer to 8.9 M, had a pH of 7.3. The neutralized formamide was added to the ribosomes at 0°, slowly, with good stirring, to a final concentration of 8.9 M (40% by volume). After standing for 30 min at 0° the mixture was dialyzed in the Zeineh dialyzer for 2.5 hr against 20 mM KCl–0.2 mM $MgCl_2$–5 mM potassium phosphate, pH 7.8. The removal of formamide was checked by measuring the refractive index of the solution in an Abbé refractometer. The solution was layered, in 6-ml portions, over 3 ml of 0.3 M sucrose containing the same buffer, and centrifuged for 1 hr at 150,000*g*. The solution above the sucrose was removed in 1- to 2-ml portions, and the samples of low absorbancy at 260 nm (usually the top 5 ml) were pooled. This material has been called the formamide extract. Unless it was to be used immediately, or fixed, one-third volume of 40% sucrose was added, and the mixture was frozen rapidly in a Dry Ice–alcohol bath and stored at −20°.

Active large subunits were isolated by the 2.7 M urea procedure of Petermann and Pavlovec (1971) and concentrated by sedimenting for 18 hr at 78,000*g* in 25 mM KCl–1 mM dithiothreitol–2.3 mM $MgCl_2$–1 mM potassium phosphate, pH 7.5. The pellets were suspended in the bottom layers of supernatant. This solution, containing 19 mg of subunits in 7 ml, was adjusted to contain 100 mM KCl, dialyzed for 3 hr against the 0.1 mM $MgCl_2$ buffer, and treated with formamide as described above.

To separate the RNP from the remaining ribosomal subunits and the tRNA the formamide extract was concentrated to 4 ml by further dialysis against the same buffer, in a Schleicher–Schuell membrane supported in a 500-ml vacuum flask, under reduced pressure. The outside buffer was stirred magnetically, and the inside solution was mixed by slowly bubbling nitrogen. The material was then fractionated on G-200 Sephadex, in the same buffer. The eluate was concentrated as described above or dialyzed against 30 mM ammonium formate and lyophilized.

The formamide extract was fixed with formaldehyde by adding 0.25 volume of 2 M formaldehyde–20 mM KCl–0.2 mM $MgCl_2$–5 mM potassium phosphate, pH 7.4. The mixture was dialyzed overnight against the same formaldehyde solution, then concentrated to 4 ml by dialysis against 0.1 M formaldehyde–100 mM KCl–0.2 mM $MgCl_2$–3 mM potassium phosphate, pH 7.4.

Polyacrylamide gel electrophoresis was carried out as described by Peacock and Dingman (1967), except that dimethylaminopropanol was replaced in the gel by 0.08% tetramethylethylenediamine. Samples of 100 μl, containing 0.3 A_{260} unit of RNA, were applied to 1-cm slots in 10 × 17 × 0.6 cm slabs of 10% gel. Separate segments were stained with Methylene Blue for RNA and with Coomassie Blue for protein. The gels were photographed on Polaroid 46L film with an orange filter and the films were scanned in a densitometer. The relative amounts of RNP and 5S RNA were calculated from the areas under the peaks. To dissociate the RNP 100 μl of formamide extract was mixed with 20 μl of 1% sodium dodecyl sulfate before the sample was put into the gel (Dahlberg *et al.*, 1969). The lyophilized RNP was dissolved in 0.2 ml of 1% sodium dodecyl sulfate, 1% mercaptoethanol, and the molecular weight of the protein was measured by gel electrophoresis in sodium dodecyl sulfate as described by Weber and Osborn (1969), by comparison with four marker proteins: phosphorylase A subunit, mol wt 94,000; bovine serum albumin, 67,000; α-chymotrypsinogen, 27,500; and myoglobin, 17,200.

Samples of fresh or formaldehyde-fixed RNP, concentrated to 1.0 A_{260} unit/ml, were examined in the analytical ultracentrifuge at 5° and 59,780 rpm, with ultraviolet optics. Fixed RNP samples were also examined by isodensity equilibrium centrifugation in CsCl. Solutions with an initial density of 1.55 g/cm^3 were centrifuged at 52,000 rpm for 18 hr at 25°, and the RNA and protein content of the material was calculated from the band position, as described by Hamilton (1971).

Results

Electrophoretic Patterns of Formamide Extracts. When the extract of whole ribosomes was examined by gel electrophoresis, the RNA stain (Figure 1B) showed a tRNA band, a 5S RNA band, and a sharp slower band that always traveled 35% as far as the tRNA. The protein stain (1C) showed the same sharp band plus a small amount of slightly slower protein. After treatment with sodium dodecyl sulfate (Figure 1D) the slow RNA band disappeared and the 5S RNA band became more intense. From these results we concluded that the slow band represented a complex of 5S RNA and protein. An extract prepared from isolated large subunits (Figure 1F) showed the RNP band and the 5S RNA band, but no tRNA. With the protein stain (1E) it showed only a single band.

Stability. The RNP complex was unstable, being easily converted to free 5S RNA and protein. It could be stored at −20° in 10% sucrose. Concentration by precipitation with zinc (Raj and Rao, 1969) or ethanol, or by lyophilizing, dissociated it. To study its stability samples of formamide extract were mixed with various buffers, stored at 5° for 3 or 4 days, and examined by gel electrophoresis and staining

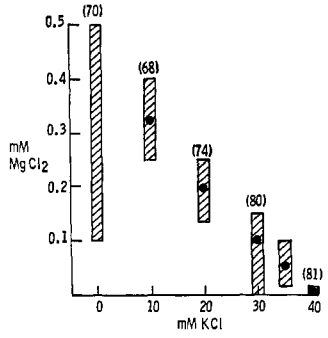

FIGURE 2: The effect of KCl and $MgCl_2$ on the stability of the RNP at 5°, in 5 mM potassium phosphate, pH 7.8. The points show the optimal $MgCl_2$ concentration for each KCl concentration, and the figures in parentheses show the percentage of RNP remaining after 4 days. The bars show the ranges over which the percentage of RNP was within 5% of the best value.

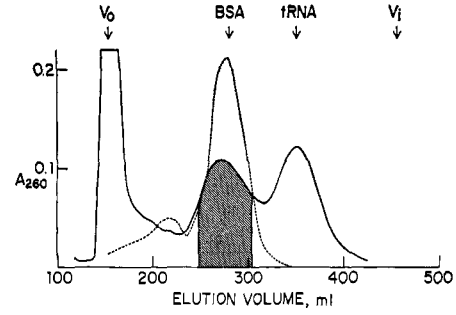

FIGURE 3: The fractionation of the formamide extract on G-200 Sephadex in 30 mM KCl–0.3 mM $MgCl_2$–3 mM potassium phosphate, pH 7.8 (solid line). The hatched area indicates the elution of the RNP. The dotted line shows the elution of bovine serum albumin, in a separate experiment with the same column. V_0, void volume; BSA, albumin monomers; V_i, inclusion volume, as measured by the elution of *N*-2,4-dinitrophenyl-L-alanine.

for RNA. At pH 7.8 a KCl concentration above 50 mM was unfavorable, and NaCl was worse. The stability was unchanged when the pH was lowered to 6.5 with cacodylate buffer, but was markedly decreased in acetate at pH 5.0. In 40 mM KCl–1 mM EDTA at pH 7.8, 3 buffers, 5 mM potassium phosphate, 3 mM Tris, and 5 mM triethanolamine, gave similar results.

A series of experiments was carried out in 5 mM potassium phosphate, pH 7.8, containing different amounts of KCl and $MgCl_2$. With no KCl, the complex was equally stable over a range of $MgCl_2$ concentrations (Figure 2). As the KCl concentration was increased from 10 to 35 mM the optimal amount of $MgCl_2$ decreased, and in 40 mM KCl the complex was most stable when all the Mg^{2+} was sequestered by EDTA. The ranges of Mg^{2+} concentration shown in Figure 2 were obtained from a series of experiments on different preparations. When all the optimal solvents were compared on the same preparation (figures in parentheses), the best value, 81%, was obtained in 40 mM KCl with EDTA. After Sephadex fractionation the RNP was much less stable; half the material that had emerged from the column in the RNP fraction was converted to free 5S RNA in only 1 day.

Since the Mg^{2+} concentration needed to reassociate the RNP with the rest of the large subunit may be much higher than the levels compatible with long-term stability, the short-term effect of increased $MgCl_2$ was examined. When the complex was kept in 20 mM KCl–5 mM potassium phosphate, pH 7.8, for only 60 min before analysis the $MgCl_2$ concentration could be varied from 0.1 to 3 mM with no decrease in the percentage of RNP.

Sephadex Fractionation. When formamide extracts from whole ribosomes were fractionated on G-200 Sephadex (Figure 3) the subunits were eluted at the void volume, 152 ± 3 ml, and the tRNA at 300–400 ml. The RNP was eluted just ahead of bovine serum albumin monomers, at a volume that would correspond to a protein of molecular weight about 71,000. The albumin has a density of 1.362 g/cm³ (Dayhoff *et al.*, 1952), and the density of the RNP is 1.568 g/cm³, as shown below; after correcting for this difference in weight to volume ratio the molecular weight of the RNP is about 81,000. On electrophoresis the RNP fraction showed the RNP band plus some free 5S RNA. The free RNA should have been eluted just ahead of the tRNA (Wilson and Quincey, 1969). In this experiment the formamide was removed by dialysis against buffer containing only 10 mM KCl. Following the stability studies, extracts were made in 20 mM KCl–0.2 mM $MgCl_2$–5 mM potassium phosphate, pH 7.8. When more electrolyte was present the ribosomal subunits sedimented faster and were more efficiently removed from the formamide extract. After Sephadex fractionation in this buffer the recovery of 5S RNA in the RNP fraction was about 70% of the theoretical amount. Material fixed with formaldehyde and fractionated in 100 mM KCl gave an elution pattern like that shown in Figure 3.

Characterization. In velocity sedimentation the 5S RNA and RNP boundaries were not resolved; the RNP obtained by Sephadex fractionation of unfixed material gave a single boundary with $s_{20,w}$ of about 6 S. For two samples that were fixed before Sephadex fractionation and contained only 5% of free 5S RNA, $s_{20,w}$ was 6.8 S. In CsCl three samples of fixed RNP banded at a density of 1.568 g/cm³. This corresponds to a partial specific volume of 0.64 and an RNA content of 48.5%; since the molecular weight of the 5S RNA is 40,000, the RNP should have a molecular weight of 83,000.

When the lyophilized RNP fraction was examined by electrophoresis in sodium dodecyl sulfate, it showed a strong protein band with a molecular weight of 41,000. A complex of one molecule of 5S RNA and one protein chain would have a molecular weight of 81,000.

Discussion

The 5S RNA of the rat liver ribosome is associated with a protein of mol wt 41,000. The size of this complex was determined in two ways. On Sephadex it was consistently eluted at a volume slightly less than that for bovine albumin monomers, and had an apparent molecular weight of 81,000. The accuracy of this determination would, however, be affected by differences in the shape and hydration of the two particles. With M = 81,000, s = 6.8 S, and $\bar{V}$ = 0.64 the frictional ratio (Edsall, 1953) would be 1.38. Since this is slightly higher than the frictional ratio of bovine albumin, 1.30 (Phelps and Putnam, 1960), the RNP is more hydrated or more asymmetrical. Since this would tend to reduce its relative elution volume, and give an apparent molecular weight slightly greater than the true value, the Sephadex measurement does show that each RNP particle can contain only one protein chain. The second estimate of molecular weight is based on the RNA content of the particle, calculated from its buoyant density in CsCl. Although the density is affected by hydra-

tion and specific interaction with gradient ions, the results obtained on a variety of ribosomal particles agree closely with chemical analyses (Hamilton, 1971). The value obtained for the RNP, 48.5% RNA, again is compatible with the presence of only one protein chain per particle.

Since the protein has an isoelectric point near pH 7 (A. Pavlovec, unpublished results) the complex is much less stable than a nucleohistone, and is easily dissociated by moderate concentrations of KCl or NaCl. The decrease in stability at very low cation concentration may reflect an unfolding of the RNA. Magnesium is not essential for stability, since the complex is not dissociated in EDTA, and too much $MgCl_2$ may be detrimental. The effect of higher Mg^{2+} concentration may be due to replacement, on the RNA phosphates, of protein by Mg^{2+}; electrostatically bound extraneous proteins can be removed from ribosomes by Mg^{2+} (Beeley *et al.*, 1968). Reticulocyte RNP is also unstable (Lebleu *et al.*, 1971).

The 5S RNA is also detached from the large subunit of the liver ribosome in 5 M urea (M. L. Petermann, A. Pavlovec, and M. G. Hamilton, in preparation) or when the Mg^{2+} is chelated with EDTA (Hamilton *et al.*, 1971). Both extracts contain the RNP complex (Peterman *et al.*, 1971) but the ratio of RNP to free 5S RNA is less than in formamide extracts. Since similar RNPs are obtained by removing Mg^{2+} and by exposure to formamide and urea, which appear to act by breaking hydrogen bonds (Petermann and Hamilton, 1968), the 5S RNA and its protein seem to be closely associated within the large subunit. A 5S RNP has also been seen in electrophoretic patterns of extracts from yeast ribosomes (Mazelis, 1970) and similar bands appear in electrophoretic patterns made from kidney and brain material (Peacock and Dingman, 1967). A complex containing 5S RNA and a protein of mol wt 45,000 has been extracted from reticulocyte polysomes with EDTA (Lebleu *et al.*, 1971), and Blobel (1971) has found a molecular weight of 35,000 for the liver protein.

Aside from the RNP protein little or no structural protein is detached from the large subunit by formamide treatment. No other acidic protein appears in Figure 1F. On electrophoresis in sodium dodecyl sulfate the only strong band was the RNP protein. Many very faint bands were also seen, but since this extract had not been freed of subunits by Sephadex fractionation, traces of subunit proteins would have been extracted by the sodium dodecyl sulfate. Since the residual particle still had a density of 1.62 g/cm^3 (M. L. Petermann, A. Pavlovec, and M. G. Hamilton, in preparation), close to that of the active subunit, 1.614 g/cm^3 (Hamilton *et al.*, 1971), similar amounts of RNA and protein had been removed. The residual proteins gave a complex pattern on electrophoresis in sodium dodecyl sulfate; the only difference from the pattern of the active subunit was the absence of one minor band, of molecular weight about 40,000 (A. Pavlovec, unpublished results). A single protein was also removed from the large subunit of the *Escherichia coli* ribosome when the 5S RNA was extracted with 0.5 M NH_4Cl (Siddiqui and Hosokawa, 1968), and from the reticulocyte large subunit treated with EDTA (Blobel, 1971). Attempts to reattach 5S RNA and form active large subunits have been generally unsuccessful, except for the complete reconstitution of large subunits from *Bacillus stearothermophilus* (Nomura and Erdmann, 1970) and from *E. coli* (Maruta *et al.*, 1971). The protein in the RNP complex may be essential for reassembly. In preliminary experiments, when the Mg^{2+} concentration was gradually increased to 3 mM over a 1-hr period 10–20% of the formamide-treated large subunits combined with the RNP to give active particles.

Acknowledgment

We thank Mrs. Concepcion Kane and Mrs. Cecile Bolisay for valuable technical assistance.

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