Studies on a Nucleoprotein Prepared from Rat Liver Polysomes by Digestion with T₁ Ribonuclease

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1. Treatment of rat liver polysomes in a buffer containing 2.5 mM-magnesium chloride with T₁ ribonuclease at a concentration of 330 units/ml. of reaction medium at 37° for 2 hr. leads to the production of an insoluble nucleoprotein. 2. On the bases of analysis for protein and RNA and of u.v.-absorption spectra the nucleoprotein appears to have lost approx. 60% of the structural RNA originally present in the ribosome. Degradation of ³H-labelled polysomes (structural RNA labelled with orotic acid) with T₁ ribonuclease leads to nucleoprotein preparations retaining approx. 30% of the radioactivity originally present in the polysomes. By means of sucrose-density-gradient centrifugation it is shown that the nucleoprotein preparations are free of single 73s ribosomes and ribosomal subunits. No evidence for the presence of 28s and 18s structural RNA was obtained on examination of extracted nucleoprotein-particle RNA by means of sucrose-density-gradient centrifugation. 3. Digestion of washed polysomes carrying ¹⁴C-labelled nascent peptide chains with T₁ ribonuclease gives a nucleoprotein particle that retains approx. 70% of the original labelled chains. Treatment of labelled nucleoprotein particles with 1 mM-puromycin in the absence of transfer factors releases 20% of the labelled chains. Addition of GTP (0.48 μ mole) increases this release to 37%. 4. Treatment of nucleoprotein particles carrying ¹⁴C-labelled peptide chains with either EDTA (50 mM) or ammonium chloride (0.5 M) brings about a small release of labelled material (approx. 15%). 5. Disruption of nucleoprotein particles carrying ¹⁴C-labelled peptide chains with either sodium dodecyl sulphate or 2 M-lithium chloride, followed by addition of transfer RNA as marker and chromatography on Sephadex G-200, show in both cases that considerable amounts of labelled peptide material move well ahead of the added transfer RNA marker. Further, if nucleoprotein particles carrying labelled peptide chains are treated with 0.3 M-potassium hydroxide at 20° for 24 hr., neutralized to pH 7.6, and then chromatographed on Sephadex G-200, the labelled peptide material moves much closer to the added transfer RNA marker. These results suggest that a proportion of the nascent ¹⁴C-labelled peptides on the nucleoprotein are attached to transfer RNA or large fragments of transfer RNA. 6. [³H]Polyuridylic acid binds to nucleoprotein particles in 1 mM-magnesium chloride. The rate of binding is rapid when measured at 20°.

In the *Escherichia coli* ribosomal system, labelled peptide chains attached to t-RNA* are released from the ribosome in solutions of low Mg²⁺ concentration or by means of EDTA (Cannon, 1967). Release of peptidyl-t-RNA from rat liver polysomes requires treatment with EDTA (Wettstein & Noll, 1965). Little, however, is known about the mode of attachment of peptidyl-t-RNA to ribosomes, and whether the area of attachment is on the surface of, or buried within, the ribosome. In this respect,

* Abbreviations: t-RNA, transfer RNA; m-RNA, messenger RNA; polyU, polyuridylic acid.

Rich, Eikenberry & Malkin (1966) have reported on the mild digestion with the proteolytic enzymes trypsin and Pronase of reticulocyte ribosomes carrying labelled peptide chains, and from their results have suggested that the growing point of the peptide chain and presumably the t-RNA to which it is attached are deeply buried within the ribosome. Pestka (1967) has shown that the aminoacyl linkage of phenylalanyl-t-RNA bound to 70s ribosomes is protected against alkaline hydrolysis. On the other hand, hydrolysis occurs when the phenylalanyl-t-RNA is bound to the 30s ribosomal subunit.

Addition of the 50s subunit to the 30s subunit-phenylalanyl-t-RNA complex confers protection on the aminoacyl linkage.

My approach to studying the problem of whether peptidyl-t-RNA is on the surface or buried within the ribosome has been to digest polysomes with T_1 ribonuclease under various conditions and to examine the digested particles to see whether they still carry peptide chains or not. By this approach I have been able to prepare reproducibly a nucleoprotein from polysomes that has a number of interesting properties, which form the contents of the present paper and which appear to support the idea that a proportion of the peptidyl-t-RNA is deeply buried within the ribosome.

MATERIALS AND METHODS

Chemicals and enzymes. ATP (disodium salt), GSH, phosphoenolpyruvate (tricyclohexylammonium salt) and pyruvate kinase were obtained from C.F. Boehringer and Soehne G.m.b.H., Mannheim, Germany. LiCl was supplied by the Fisher Scientific Co., Pittsburg, Pa., U.S.A. T_1 ribonuclease (crystalline, B grade) was obtained from Calbiochem, Los Angeles, Calif., U.S.A. Sephadex G-200 was supplied by Pharmacia, Uppsala, Sweden. Dodecyl sulphate (sodium salt) was obtained from Serva, Heidelberg, Germany. L(-)-Phenylalanine was supplied by E. Merck A.-G., Darmstadt, Germany. ^{14}C -labelled yeast-protein hydrolysate (1500 $\mu\text{C}/\text{mg.}$), [^3H]phenylalanine (1000 $\mu\text{C}/\text{m-mole}$), [^3H]orotic acid (16.8 $\text{c}/\text{m-mole}$) and [^3H]polyU (7.85 $\text{mc}/\text{m-mole}$) were obtained from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. Sodium deoxycholate was supplied by General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A. PolyU (ammonium salt, unlabelled) was supplied by Miles Laboratories Inc., Elkhart, Ind., U.S.A. All other reagents and chemicals used were of A.R. grade.

Preparation of cell particles and enzyme fractions. Polysomes were prepared from homogenates of rat liver in medium A [containing (final concentrations): sucrose (0.25 M), MgCl_2 (5 mM), KCl (25 mM) and tris-HCl buffer, pH 7.6 (50 mM)] by the method of Wettstein, Staehelin & Noll (1963) as described for the Spinco no. 30 rotor by Hawtrey & Nourse (1966). They were finally suspended in medium A and stored at 0° .

pH 5 fraction. This was prepared from a sample of cell sap as described by Hawtrey & Nourse (1966). It was suspended in medium A and stored at -15° .

Preparation of nucleoprotein. A suspension of polysomes in medium A was diluted with the same medium so that, on dilution of a 0.1 ml. portion of the final suspension to 3.0 ml., it had an E_{260} of 1.5. To 4 ml. of diluted polysome suspension (in medium A) was added 2 ml. of T_1 ribonuclease solution (2000 units in water; units of enzyme activity are those defined by Takahashi, 1961). After careful mixing, the reaction mixture was incubated for 2 hr. at 37° with occasional stirring. At the end of this time the nucleoprotein particles had settled out as a fine insoluble precipitate. The reaction mixture was then cooled in ice, and after mixing was centrifuged at 1400g (in an International centrifuge) for 15 min. The clear supernatant was decanted and the pellet of nucleoprotein particles suspended in medium A

(by stirring with a glass rod) and then centrifuged again as described above. Normally the centrifugation-washing procedure with medium A was repeated three times. The nucleoprotein particles were finally suspended in 6 ml. of medium A and stored at 0° . Alternatively, the nucleoprotein particles were suspended in the buffer appropriate to the particular experiment.

Analysis of nucleoprotein prepared from ^3H -labelled polysomes. Rats were given an intraperitoneal injection of [^3H]orotic acid (30 $\mu\text{C}/\text{animal}$) and killed 14 hr. after administration of the labelled material. Polysomes (labelled in their structural RNA) were prepared from homogenates in medium A as described above. Digestion of the washed labelled polysomes with T_1 ribonuclease was carried out as described for the preparation of the nucleoprotein. Both labelled polysomes and washed nucleoprotein particles were treated with cold 5% (w/v) trichloroacetic acid and the resulting precipitates washed three times with cold 5% trichloroacetic acid by repeated centrifugation. They were finally transferred to Millipore filters, and washed with cold 5% trichloroacetic acid ($3 \times 5 \text{ ml.}$). Results obtained were: polysomes (3.80 E_{260} units), 3672 c.p.m.; nucleoprotein (derived from 3.80 E_{260} units of polysomes), 1022 c.p.m.

System for incorporation of ^{14}C -labelled amino acids into polysomes. The reaction medium used for incorporation of labelled amino acids contained: tris-HCl buffer, pH 7.6 (50 mM); KCl (25 mM); MgCl_2 (8 mM); sucrose (0.24 M); ATP (potassium salt, pH 7.6 (30.0 μmoles); GTP (potassium salt, pH 7.6 (11.6 μmoles); phosphoenolpyruvate (tricyclohexylammonium salt) (125 μmoles); phosphoenolpyruvate kinase (700 $\mu\text{g.}$); GSH, pH 7.6 (70 μmoles); complete ^{14}C -labelled yeast-protein hydrolysate (15 μC); polysomes (14–18 mg. of protein); pH 5 enzyme (12 mg. of protein); NH_4Cl (11 mM). The final volume of the reaction medium was 15.0 ml. Incubation was carried out for 12 min. at 37° . The labelled polysome suspension was layered over 0.5 M-sucrose (containing medium A buffer) and centrifuged at 99000g for 2.5 hr. in the Spinco no. 30 rotor. The polysomal pellet was taken up in medium A and the washing-centrifugation procedure with 0.5 M-sucrose repeated once again. The resulting pellet was taken up in medium A, cleared of debris by low-speed centrifugation and the clear suspension of polysomes kept at 0° .

Release of perchloric acid-soluble fragments from polysomes on digestion with T_1 ribonuclease. To a sample of polysomes (14.0 E_{260} units) in medium A (quantities are defined in terms of E_{260} units as given in the preparation of nucleoprotein particles) was added T_1 ribonuclease (10–330 units/ml. of incubation medium). After mixing, samples were incubated at different temperatures for various times. Samples (0.25 ml.) of reaction medium were removed at different time-intervals and mixed with 0.1 ml. of bovine serum albumin (1 mg.) and 5 ml. of cold 0.4 M- HClO_4 . After 10 min. in ice, samples were centrifuged at 1400g for 15 min., and the E_{260} values of the clear supernatants measured to determine acid-soluble nucleotide material.

Measurement of radioactivity. Depending on the type of experiment, samples were processed in a number of ways. Certain samples were washed by Millipore filtration with Nirenberg buffer, tris-acetate, pH 7.2 (0.10 M), magnesium acetate (20 mM) and KCl (50 mM) (Nirenberg & Leder, 1964), or with medium A. Other samples were washed with cold 5% (w/v) trichloroacetic acid by low-speed centrifugation before transfer to Millipore filters (HA Millipore filter, 25 mm.

Fig. 1. Rate of release of HClO₄-soluble nucleotides and oligonucleotides from polysomes undergoing digestion with T₁ ribonuclease, at various concentrations of enzyme and MgCl₂. ●, T₁ ribonuclease concn. 100 units/ml. of reaction medium, MgCl₂ (5 mM) and at 37°; ○, T₁ ribonuclease concn. 100 units/ml., MgCl₂ (15 mM) and at 37°; ●, T₁ ribonuclease concn. 330 units/ml., MgCl₂ (2.5 mM) and at 37°; —, T₁ ribonuclease concn. 100 units/ml., MgCl₂ (5 mM) and at 0°. Details of the method of analysis are given in the Materials and Methods Section.

diam., 0.45 μ m. pore size) followed by washing with trichloroacetic acid. Filters were counted either in toluene containing 0.5% (w/v) of 2,5-diphenyloxazole and 0.03% (w/v) of 1,4-bis-(5-phenyloxazol-2-yl)benzene, or in Bray's (1960) solution.

Protein and RNA. These were determined as described by Hawtrey, Schirren & Dijkstra (1963).

RESULTS

Time-course of release of perchloric acid-soluble products from polysomes undergoing digestion with T₁ ribonuclease at 37° and at different Mg²⁺ and enzyme concentrations. Fig. 1 shows that, at a concentration of 100 units/ml., T₁ ribonuclease brought about the release of perchloric acid-soluble oligonucleotides and nucleotides at a fairly constant rate. Very little difference in rate was observed at either 3 mM- or 15 mM-Mg²⁺. However, at the higher Mg²⁺ concentration a heavy precipitate of degraded

ribosomes was observed at 3 hr. of incubation, whereas this phenomenon was only noted at 4.5 hr. with the lower Mg²⁺ concentration. Increase of the T₁ ribonuclease concentration to 330 units/ml. of incubation medium at 2.5 mM-Mg²⁺ brought about a much more rapid release of perchloric acid-soluble fragments from polysomes and resulted in the precipitation of an insoluble nucleoprotein at 2 hr. of incubation, with the supernatant phase of the incubation medium appearing completely clear. In these experiments, 14.0 E₂₆₀ units of polysomes were used (see the Materials and Methods section). In the preparation of the nucleoprotein (Fig. 1, ●), a total of 9.5 E₂₆₀ units were released as perchloric acid-soluble material. This corresponds to 67% loss of 260 nm-absorbing material from the polysomes during their conversion into the nucleoprotein. In another experiment nucleoprotein particles were prepared from polysomes, and the resulting aqueous phase remaining after removal of the nucleoprotein by low-speed centrifugation was measured directly at 260 nm. (perchloric acid was not used). A 68% loss of E₂₆₀ units was found.

Results obtained by the two methods agreed very well. However, no correction was made for changes in hyperchromicity of the E₂₆₀ absorbing material produced by digestion with T₁ ribonuclease.

General properties of the nucleoprotein precipitated after 2 hr. of digestion with T₁ ribonuclease at 37°. Nucleoprotein particles were prepared in a medium containing 2.5 mM-magnesium chloride and 330 units of enzyme/ml. They were easily resuspended in medium A, and could be sedimented at the relatively low speed of 1400 g for 15 min., as opposed to the high speed needed to sediment polysomes. Washing of the particles was carried out in medium A by repeated centrifugation at low speed.

Ultraviolet-absorption spectra of both the nucleoprotein particle and polysomes are shown in Fig. 2. Nucleoprotein particles gave an E₂₆₀/E₂₈₀ ratio 1.14 and an E₂₄₅/E₂₆₀ ratio 0.93. Polysomes gave a value of 1.80 for the E₂₆₀/E₂₈₀ ratio and 0.63 for the E₂₃₅/E₂₆₀ ratio. Some variation was observed in the values for nucleoprotein particles prepared from different batches of polysomes. Analysis for RNA and protein gave an RNA/protein ratio 0.52 for nucleoprotein particles and 1.30 for polysomes. The value for the nucleoprotein was somewhat variable for different preparations, as expected. Both the chemically determined RNA/protein ratio for the nucleoprotein and the amount of RNA lost from polysomes in preparing the nucleoprotein, as determined by u.v.-absorption measurements of perchloric acid-soluble fragments, indicated that the ribosome loses approx. 60% of its structural RNA during transformation into the nucleoprotein. As these are approximate values, a more accurate determination of the RNA lost from polysomes

Fig. 2. Ultraviolet spectra of polysomes and nucleoprotein particles. All spectra were determined in medium A. A, Polysomes ($0.54 E_{260}$ unit); B, nucleoprotein particles ($0.41 E_{260}$ unit).

during formation of the nucleoprotein particle was carried out with polysomes previously labelled with [^3H]orotic acid.

The results of such an experiment indicate that the polysomes have lost 73% of their radioactivity (labelled structural RNA), and hence the nucleoprotein particle contains approx. 30% of the original ribosome RNA (see the Materials and Methods section).

Confirmation of the absence of single 73s ribosomes and 50s and 30s subunits was obtained by sucrose-density-gradient centrifugation of nucleoprotein preparations. Fig. 3(a) shows the profiles for polysomes incubated for 2 hr. at 37° , and for a mixture of nucleoprotein particles and released nucleotide material that resulted from treating polysomes with T_1 ribonuclease (330 units/ml.) for 2 hr. at 37° . The nucleoprotein preparation does not contain any ribosomes or ribosomal subunits. A large clear gelatinous pellet of nucleoprotein particles was observed at the bottom of the centrifuge tube after the centrifugation. The result of treating polysomes with T_1 ribonuclease (100 units/ml.) for 2 hr. at 37° followed by sucrose-density-gradient centrifugation is shown in Fig. 3(b). At

Fig. 3. Sucrose-density-gradient centrifugation of polysomes and nucleoprotein preparations. Linear sucrose gradients (27 ml.; 16–34%, w/v) contained tris-HCl, pH 7.6 (50 mM), and MgCl_2 (5 mM). Centrifugation was carried out in the Spinco SW 25.1 rotor at 25 000 rev./min. (90 000 g) for 4.5 hr. Fractions (1 ml.) were collected. (a) —, Normal polysomes preincubated for 2 hr. at 37° , then cooled in ice before centrifugation; ----, nucleoprotein particles resulting from treating polysomes with T_1 ribonuclease (330 units/ml. of incubation medium) in a buffer containing 2.5 mM- MgCl_2 for 2 hr. at 37° . (b) —, Degraded polysomes resulting from treating polysomes with T_1 ribonuclease (100 units/ml. of incubation medium) at 2.5 mM- MgCl_2 for 2 hr. at 37° .

this concentration of enzyme, considerable amounts of ribosomal material remain.

Examination of the structural RNA remaining in nucleoprotein was carried out either by extracting the washed particles with phenol or by means of 2 M-lithium chloride. In either case similar results were obtained. Fig. 4 shows the results of running control 28s and 18s ribosomal RNA, in comparison with the structural RNA present in the nucleoprotein, on sucrose density gradients. No evidence for the presence of normal ribosomal RNA in the nucleoprotein preparation was obtained.

Binding of nascent ^{14}C -labelled peptide chains to nucleoprotein preparations. Polysomes were labelled with ^{14}C -labelled peptide chains in a complete incorporation system as described in the Materials and Methods section. The labelled polysomes were then treated with T_1 ribonuclease under standard

Fig. 4. Sucrose-density-gradient centrifugation of structural RNA extracted from polysomes and nucleoprotein particles. Linear sucrose gradients (27 ml.; 5–20%, w/v) contained potassium acetate, pH 5.2 (10 mM), NaCl (50 mM) and EDTA (5 mM). Centrifugation was carried out in the Spinco SW 25.1 rotor at 24000 rev./min. (85000g) for 14 hr. Fractions (1 ml.) were collected, diluted to 2 ml. with water and then read at 260 nm. (a) —, Structural RNA from polysomes, 3.50 E_{260} units. (b) —, RNA extracted from nucleoprotein particles by the LiCl method, 3.30 E_{260} units.

conditions to produce the nucleoprotein. Nucleoprotein preparations were found to retain a large proportion of the peptide radioactivity originally present on the polysomes. In a typical experiment the following results were obtained. The original polysome suspension gave 12047 c.p.m./mg. of protein. Treatment of a fraction of the polysome suspension with T₁ ribonuclease under standard conditions gave a nucleoprotein with 8464 c.p.m./mg. of protein. These results were calculated on the basis that no structural protein is lost in the formation of the nucleoprotein. Further, on repeated washing by low-speed centrifugation in medium A, nucleoprotein preparations lose little radioactivity and appear to retain approx. 70% of the peptide radioactivity originally on the polysomes (nucleoprotein at isolation, 8830 c.p.m./mg. of protein; nucleoprotein after third wash, 8464 c.p.m./mg. of protein). Details of the washing procedure are given in the Materials and Methods section.

Having established that the nucleoprotein preparations carry considerable amounts of nascent peptide chains, it was decided to establish whether a proportion of the radioactivity would be affected by treatment with puromycin or not. Table 1 shows the results of treating washed nucleoprotein preparations with puromycin in the absence and presence of either GTP or pH 5 enzyme or both.

Table 1. Effect of puromycin and other factors on the proportion of ¹⁴C-labelled peptide chains sedimented with nucleoprotein after 15 min. at 1400g

In the release experiments, constituents of medium A buffer were present at the concentrations used for labelling polysomes, as given in the Materials and Methods section. The following constituents were present in all incubation mixtures at the following concentrations: ¹⁴C-labelled nucleoprotein (0.14 mg. of protein); phosphoenolpyruvate (potassium salt) (5.2 μ moles); pyruvate kinase (60 μ g.); GSH (5.8 μ moles), pH 7.6. Other constituents when present were at the following concentrations; GTP (0.48 μ mole); pH 5 enzyme (1.8 mg. of protein); puromycin (1 mM). Reactions were run for 20 min. at 37°. Samples were cooled in ice, and processed for counting by (a) centrifuging at 1400g for 15 min. followed by washing of the precipitates by the Millipore procedure with medium A (4 $\times$ 5 ml.) or (b) direct washing by the Millipore procedure with medium A.

System	Release of ¹⁴ C-labelled peptide chains [c.p.m./mg. of protein (nucleoprotein)]	
	Washing directly by Millipore procedure	Washing by centrifugation followed by washing by Millipore procedure
Nucleoprotein	21022	20671
Nucleoprotein + puromycin	21326	16585
Nucleoprotein + puromycin + GTP	20907	13128
Nucleoprotein + puromycin + GTP + pH 5 enzyme	17950	13035

The assay described in Table 1 measures the effect of puromycin and other factors on the proportion of ¹⁴C-labelled peptide chains sedimented with the nucleoprotein material after 15 min. at 1400g, and hence may differ from the release assay used for normal protein-synthesizing systems. In the absence of either GTP or pH 5 enzyme, 1 mM-puromycin released 20% of the labelled peptide chains. Addition of GTP (0.48 μ mole) increased the release to 37%. The presence of added pH 5 enzyme did not enhance the amount of peptide released in the presence of puromycin and GTP.

The effect of treating nucleoprotein preparations carrying ¹⁴C-labelled peptide chains with EDTA or ammonium chloride was also studied to determine how tightly the peptide chains are attached to the nucleoprotein. Results in Fig. 5 show that 50 mM-EDTA, pH 7.6 at 0° brings about a rapid release of approx. 15% of the labelled chains on the nucleoprotein. Although not shown, an identical rate and amount of released ¹⁴C-labelled peptide was observed after treatment with EDTA at 37°. No

Fig. 5. Effect of EDTA and NH_4Cl on ^{14}C -labelled peptides bound to nucleoprotein particles. Nucleoprotein particles (0.1 ml. samples containing 1850 c.p.m.) were incubated with either 50 mM-EDTA (○) or 0.5 M- NH_4Cl (●) in 25 mM-tris-HCl, pH 7.6 for various times at 0°. All samples were centrifuged at 1400g for 15 min., and portions of the supernatants added to Bray's (1960) solution for counting. —, Control input radioactivity.

release of labelled material from the nucleoprotein was observed on incubation in 25 mM-tris-hydrochloric acid, pH 7.6. Treatment with 0.5 M-ammonium chloride gave similar results to that observed with EDTA (Fig. 5).

^{14}C -labelled peptidyl-t-RNA on nucleoprotein. To ascertain whether any of the ^{14}C -labelled peptides on the nucleoprotein are still attached to t-RNA molecules or not, the following procedure was carried out. Thoroughly washed nucleoprotein particles (in medium A) were treated in one of two ways. First, disruption of the labelled nucleoprotein with sodium dodecyl sulphate was carried out by the method of Gilbert (1963). A sample of t-RNA was then added as marker, and the complete mixture was subjected to chromatography on a Sephadex G-200 column (1 cm. $\times$ 33 cm.) by the method described by Bresler *et al.* (1966). Fig. 6 shows the results of such an experiment. In agreement with the results obtained by Bresler *et al.* (1966), it was found that a large proportion of the peptide radioactivity was eluted well before the added t-RNA marker. According to Bresler *et al.* (1966) this material represents polynucleotide-peptide material. Further experiments were carried out in which nucleoprotein particles carrying ^{14}C -labelled peptide chains were subjected to alkaline hydrolysis in

Fig. 6. Chromatography of ^{14}C -labelled peptides attached to nucleoprotein particles on Sephadex G-200 after treatment with sodium dodecyl sulphate. To 0.75 ml. of a nucleoprotein preparation (10640 c.p.m.) were added 2.5 E_{260} units of t-RNA (marker) and 3.5 ml. of a solution containing sodium dodecyl sulphate (0.25% w/v), NaCl (0.1 M) and tris-HCl, pH 7.6 (10 mM). The complete mixture was chromatographed on a Sephadex G-200 column (1 cm. $\times$ 33 cm.) at 12° with the above sodium dodecyl sulphate solution as eluent. Fractions (1.5 ml.) were collected, diluted to 2 ml. with water, read at 260 nm. and then counted in Bray's (1960) solution. —, E_{260} ; ●, radioactivity; ○, t-RNA marker run separately.

0.3 M-potassium hydroxide at 20° for 24 hr. At the end of the hydrolysis, samples were neutralized to pH 7.6, treated with sodium dodecyl sulphate and run on Sephadex G-200 columns (1 cm. $\times$ 33 cm.) as described above. Fig. 7 shows that the peptide radioactivity then moved much closer to the added t-RNA marker. This result is very similar to that obtained by Bresler *et al.* (1966) in their studies on polynucleotide-peptides in the *E. coli* system, and suggests that the peptides on the nucleoprotein were originally attached to t-RNA or large fragments of t-RNA.

Secondly, nucleoprotein particles carrying ^{14}C -labelled peptides were treated with 2 M-lithium chloride at 0° for 16 hr. This procedure was found to disrupt the nucleoprotein, giving a precipitate of structural RNA from the nucleoprotein (9–12 S; Fig. 4) and a clear supernatant that contained 70% of the radioactivity. Chromatography of the 2 M-lithium chloride-soluble ^{14}C -labelled peptides with added t-RNA as marker on Sephadex G-200 showed that a considerable portion of the peptide radioactivity moved well ahead of the added t-RNA marker, as found with the sodium dodecyl sulphate method shown in Fig. 6.

Fig. 7. Analysis on a Sephadex G-200 column of ^{14}C -labelled peptides attached to nucleoprotein particles after preliminary treatment of labelled nucleoprotein with alkali. A sample of nucleoprotein (0.75 ml., containing 10640 c.p.m.) was treated with 0.3 M-KOH at 20° for 24 hr. The sample was then neutralized to pH 7.6, mixed with added t-RNA marker (3.0 E_{260} units) and then added to 3.5 ml. of sodium dodecyl sulphate in buffer. The entire mixture was then chromatographed on a Sephadex G-200 column (1 cm. $\times$ 33 cm.) as described for Fig. 6. —, E_{260} ; ●, radioactivity.

Table 2. Effect of $[^3\text{H}]\text{polyU}$ concentration on its binding to nucleoprotein particles at different Mg^{2+} concentrations

Nucleoprotein particles (4.4 E_{260} units) were incubated with various concentrations of $[^3\text{H}]\text{polyU}$ in a medium consisting of tris-HCl, pH 7.6 (25 mM), KCl (25 mM) and MgCl_2 at either 2.5 mM or 1 mM. Incubations were for 25 min. at 20°. At the end of the reaction, tubes were cooled in ice, diluted with 6 ml. of the incubation buffer and then centrifuged at 1400g for 10 min. Tubes were drained and carefully wiped with filter paper. The precipitates were finally washed on to Millipore filters with incubation buffer (3 $\times$ 5 ml.). Corrections were made for the binding of $[^3\text{H}]\text{polyU}$ to Millipore filters in the absence of particles.

$[^3\text{H}]\text{PolyU}$ added (μC)	Binding of $[^3\text{H}]\text{polyU}$ (c.p.m.)	
	1 mM- Mg^{2+}	2.5 mM- Mg^{2+}
0.10	13556	17360
0.20	17443	26470
0.40	28650	38680
0.60	37600	—

Binding of $[^3\text{H}]\text{polyU}$ to nucleoprotein particles. Results illustrating the binding of $[^3\text{H}]\text{polyU}$ to nucleoprotein particles are given in Table 2. Table 2 shows the effect of concentration of labelled polyU on its binding to the nucleoprotein particles at 1 mM- and 2.5 mM- Mg^{2+} . In all experiments with

$[^3\text{H}]\text{polyU}$, stringent controls were run, as this material binds firmly to Millipore filters in the absence of nucleoprotein particles or polysomes. Low-speed centrifugation of nucleoprotein particles from reaction mixtures before washing on Millipore filters was essential for removing excess of $[^3\text{H}]\text{polyU}$.

DISCUSSION

Mainly as a result of work on *E. coli* ribosomes and their subunits it is now known that aminoacyl-t-RNA is bound to the 30s subunit-m-RNA complex at the decoding site and that peptidyl-t-RNA is apparently found on both the 30s and 50s subunits (Gilbert, 1963; Noll, 1966; Jost, Shoemaker & Noll, 1968). Initiation of new peptide chains on bacterial ribosomes is a complicated process involving *N*-formylmethionyl-t-RNA as an initiator and special protein initiation factors (Clark & Marcker, 1966; Adams & Capecchi, 1966; Salas, Hille, Last, Wahba & Ochoa, 1967; Revel, Herzberg, Becarevic & Gros, 1968).

With regard to the actual mechanism and mode of attachment of aminoacyl-t-RNA and peptidyl-t-RNA to ribosomes, however, very little is known. I have approached this problem by carrying out experiments in which polysomes with ^{14}C -labelled peptide chains are digested with T₁ ribonuclease under standard conditions and have examined the resulting degraded ribosomes to see whether they still have attached labelled peptides or not. It is assumed that, if peptidyl-t-RNA or labelled peptide is still found attached to degraded ribosomes, then these entities must be deeply buried within the ribosomal structure or held in some configuration at or near the ribosomal surface such that protection is afforded against ribonuclease action.

In applying this method I have been able to prepare reproducibly a nucleoprotein from polysomes under standard conditions that retains approx. 30% of the structural RNA originally present in the ribosome. As detailed in the Results section these particles possess a number of notable properties. They are insoluble in medium A, and can be sedimented at a relatively low centrifugal force. Although retaining only 30% of their original ribosomal structural RNA, they nevertheless still have attached approx. 70% of the original ribosomal ^{14}C -labelled peptide radioactivity. A certain percentage of the peptide radioactivity is released with puromycin in the absence of added factors, and this release can be increased by supplying various factors to the system (Table 1). As puromycin is capable of a partial release of labelled peptide chains from nucleoprotein particles, I assume that these peptides must be attached to t-RNA or fragments of t-RNA in an aminoacyl linkage, and that the peptide synthetase is still

capable of functioning on the nucleoprotein. Further experiments, in which nucleoprotein particles carrying ^{14}C -labelled peptide chains were treated with either sodium dodecyl sulphate or 2M-lithium chloride, followed by chromatography on Sephadex G-200 with added t-RNA as marker, showed that a large proportion of the peptide radioactivity moved well ahead of the added marker (Fig. 6). Chromatography of nucleoprotein-associated peptide radioactivity on Sephadex G-200 after alkaline hydrolysis of the complex showed that a large proportion of the radioactivity then moved much closer to the added t-RNA marker (Fig. 7). These results (Figs. 6 and 7) appear to indicate that the labelled peptides are still attached to t-RNA or large fragments of t-RNA. Further, they also suggest that a fair proportion of the peptidyl-t-RNA is buried within the ribosomal structure.

Rich *et al.* (1966) studied the digestion of labelled reticulocyte ribosomes with trypsin and Pronase, and showed that approx. 50% of the labelled peptide chains are removed by mild digestion with these enzymes. From their results they inferred that the remaining half of the peptide chain, that which is attached to the t-RNA, is deeply buried within the ribosome and therefore shielded from proteolytic action. Pestka (1967) has reported some notable findings on the stability of the aminoacyl linkage of phenylalanyl-t-RNA in the presence of *E. coli* ribosomes and subunits. When bound to the 70s ribosome, the aminoacyl linkage is protected against mild alkaline hydrolysis. However, hydrolysis occurs when the phenylalanyl-t-RNA is bound to the 30s subunit. It is notable that, on addition of the 50s subunit to the 30s subunit-phenylalanyl-t-RNA complex, protection against alkaline hydrolysis is conferred upon the aminoacyl linkage. As stated by Pestka (1967), this suggests that the aminoacyl-t-RNA may be located in an interior part of the ribosomal structure.

Cannon, Krug & Gilbert (1963) have reported on the mild digestion of *E. coli* ribosomes with pancreatic ribonuclease. Labelled peptidyl-t-RNA was still found to be bound to 'stuck' 70s *E. coli* ribosomes after treatment with ribonuclease for 12 min. at 30°. In their work no breakdown of the

ribosome structure was observed, as evidenced by sucrose-density-gradient centrifugation.

From their work on the reconstruction of *E. coli* 70s ribosomes from 50s and 30s subunits in the presence of m-RNA and peptidyl-t-RNA, Jost *et al.* (1968) have suggested that the m-RNA and peptidyl-t-RNA move through a groove or tunnel bounded by the adjoining surfaces of the two subunits.

The results given in the present paper appear to support the idea that a proportion of the peptidyl-t-RNA is buried within the ribosomal structure. This could presumably be situated in the area formed by the joining of the two subunits, as suggested by Jost *et al.* (1968).

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