Preparation of Polyribosome Aminoacyl-Transfer Ribonucleic Acid from the Muscle of Chick Embryos

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A procedure for preparing polyribosome aminoacyl-tRNA free from contamination by supernatant aminoacyl-tRNA and free amino acids is described. Important features of the procedure are the use of acidic buffers to help protect the amino acid–tRNA linkage and the inclusion of sodium dodecyl sulphate, to inhibit ribonuclease activity. The specific radioactivity of polyribosome aminoacyl-tRNA is high within 30s and reaches a maximum in 2½ min, well ahead of polyribosome peptides which, as described by Herrmann *et al.* (1971), attain maximum specific radioactivity in about 10 min.

One of the main problems encountered in studies of the rates of protein synthesis *in vivo* is an accurate identification of the real amino acid precursor pool (see, for example, Herrmann *et al.*, 1970, 1971). There is an apparent controversy among many workers as to which pool, intracellular, extracellular, membrane-bound, or a combination of these, directly supplies amino acids for protein synthesis. Thus Kipnis *et al.* (1961), Hider *et al.* (1969, 1971) and van Venrooij *et al.* (1972, 1973) have suggested that amino acids from an extracellular source directly enter into polyribosome peptides. By contrast, other workers (Fern & Garlick, 1973; Li *et al.*, 1973) have concluded that the intracellular pool serves as precursor for protein synthesis. Airhart *et al.* (1974) presented data which they interpreted as a demonstration that both the intracellular and extracellular amino acids contribute to a pool which supplies amino acids for protein synthesis.

I believe that a clear resolution of the problem would entail analyses of the specific radioactivity of the amino acid (*a*) attached to tRNA in the cell supernatant fraction and which can be regarded as being equivalent to the free intracellular aminoacyl-tRNA, (*b*) in the plasma or in extracellular space, (*c*) attached to membranes or to molecules associated with membranes, or (*d*) attached to polyribosome tRNA. This is because any of the compartments (*a*), (*b*) and (*c*) may supply amino acids directly to polyribosome tRNA and thereby to polyribosome peptides. Airhart *et al.* (1974) have described a detailed procedure for isolating valine attached to the tRNA of the cell supernatant (S-105) fraction. In the present paper I describe a method for preparing polyribosome aminoacyl-tRNA, essentially free from contamination, from aminoacyl-tRNA of the cell supernatant fraction. I have determined the kinetics of labelling of polyribosome aminoacyl-tRNA as a first

step towards comparing the specific radioactivity of amino acids in peptide linkage and the specific radioactivity of amino acid in polyribosome aminoacyl-tRNA.

Methods

Leg muscle from 14-day chick embryos (Dekalb 171, White Leghorn) was used in this study. Unless otherwise stated, all operations were carried out at 0–4°C.

Isolation of aminoacyl-tRNA

Data from this laboratory showed that 14-day embryo muscle polyribosomes comprise about 90% of the total ribosome population and that under the experimental condition of homogenizing muscle in 0.3 M-NaCl–0.01 M-MgCl₂–0.01 M-Tris-HCl, pH 7.0 (RSB buffer), ribosomes that are not in polyribosomes do not carry any tRNA (Nwagwu & Nana, 1974). I have therefore used the term ‘polyribosome’ for a total ribosome preparation.

[³H]Serine or [³H]phenylalanine (0.1 ml, corresponding to 1.2 µg of serine or 0.721 µg of phenylalanine) was injected directly into the leg muscle. The embryo was covered with tinfoil and returned to an incubator set at 37°C. At the appropriate time, leg muscle was dissected and quickly transferred to a beaker kept on ice. Usually eight or nine embryos were injected for each time-point, yielding 8–9 g of muscle. The muscle was homogenized (1 g/ml) in 0.3 M-NaCl–0.01 M-MgCl₂–0.01 M-Tris-HCl (pH 6.8)–0.04 M-serine (or 0.04 M-phenylalanine) with a loose-fitting Dounce homogenizer. The homogenate was centrifuged at 10000g for 10 min at 0°C and the resulting supernatant further centrifuged at 200000g

for 1 h at 0°C. The polyribosome pellet was resuspended in 2 ml of 1% sodium dodecyl sulphate–0.02 M-EDTA–8 mM-serine (or 8 mM-phenylalanine)–0.02 M-sodium acetate (pH 4.4) and the volume adjusted finally to 8 ml with the same buffer. To the supernatant (S-200 fraction) 5% (w/v) sodium dodecyl sulphate was added to give a final concentration of 1%. An equal volume of phenol equilibrated with 2 mM-sodium acetate (pH 4.4)–8 mM-serine (or 8 mM-phenylalanine) was added separately to the polyribosome and S-200 fractions. The tubes were shaken at 20°C with a New Brunswick shaker for 45 min, centrifuged at 17000g for 30 min at 18°C and the aqueous extract was recovered. Then 2 vol. of 95% (v/v) ethanol was added to the extract and the tubes were left at –20°C for about 18 h. RNA was recovered by centrifugation, dissolved in 0.1 M-sodium acetate, pH 4.4, and layered on a DEAE-cellulose column equilibrated against 0.1 M-sodium acetate, pH 4.4, at room temperature. The column was washed with 250 ml of 0.1 M-sodium acetate buffer, and RNA was then eluted with 0.7 M-NaCl–0.01 M-sodium acetate (pH 4.4)–0.01 M-MgCl₂. Fractions (5 ml) were collected, and those with E_{260} higher than 0.1 were pooled. Radioactivity eluted in the fractions was monitored. The pooled sample was layered directly on a column (100 cm × 2.5 cm) of Sephadex G-75 equilibrated against 0.2 M-NaCl–0.01 M-MgCl₂–0.01 M-sodium acetate, pH 4.4. Fractions (5 ml) were collected under 30 cm hydrostatic pressure at a flow rate of 30–40 ml/h. E_{254} of the eluate was continuously monitored with an ISCO u.v.-analyser. A 1.5 ml portion was pipetted from each fraction for radioactivity detection.

The peaks were characterized by their positions of elution, their R_F values on electrophoresis with polyacrylamide gels, and by their activity, or lack of it, in amino acid acceptance (A. Hossain, unpublished work). Ribosomal RNA and 5S RNA did not accept serine, under optimal conditions (A. Hossain, unpublished work); tRNA accepted serine with a high efficiency (77 pmol of serine/ E_{260} unit of tRNA).

Determination of radioactivity in trichloroacetic acid-soluble fraction

Triplicate 0.1 ml portions were taken from the supernatant from the homogenate after centrifugation at 10000g for 10 min and were transferred to tubes containing 2 ml of 0.4 M-serine (or 0.4 M-phenylalanine). They were incubated on ice for 10 min, and 2 ml of ice-cold 20% (w/v) trichloroacetic acid–4 mM-serine (or 4 mM-phenylalanine) was added. The tubes were shaken and returned to an ice bath for another 10 min. After centrifugation at 10000g for 10 min, the supernatant was poured off carefully and the pellet re-extracted with 2 ml of ice-cold 10% trichloroacetic acid–4 mM-serine (or 4 mM-phenylalanine).

Centrifugation and extraction were repeated. The supernatants were mixed and the total radioactivity was determined.

Non-specific binding of tRNA and aminoacyl-tRNA to polyribosomes

Since it has been shown in other systems that tRNA can bind to ribosomes non-specifically (Phillips, 1970; Tai & Davis, 1972), we investigated whether the same is true under our experimental conditions. [³H]Phenylalanine or [³H]serine (0.1 ml) was injected into the leg of 14-day embryos and [³H]phenylalanyl-tRNA or [³H]seryl-tRNA was prepared as described in the appropriate section above. [³H]Phenylalanyl-tRNA or [³H]seryl-tRNA was added to muscle S-10 supernatant, incubated for 30 min at 4°C and the mixture was centrifuged at 200000g for 1 h. The pellet was thoroughly rinsed with, and resuspended in, RSB buffer and the radioactivity of the pellet and the S-200 supernatant was determined.

Non-specific binding of [³H]serine or [³H]phenylalanine to tRNA

[³H]Serine or [³H]phenylalanine (each 50000 d.p.m.) was added to polyribosome and S-200 supernatant (1.5 mg of protein of each), incubated at 4°C for 30 min, and tRNA was extracted and purified by chromatography on DEAE-cellulose and Sephadex columns. The radioactivity of the RNA fraction eluted after DEAE-cellulose chromatography and of the tRNA peak eluted after Sephadex G-75 chromatography was determined.

Radioactivity determination

Radioactivity was determined in the presence of 10 ml of PCS scintillation solution with a Packard Tri-Carb liquid-scintillation spectrometer.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Materials

All chemicals were of analytical grade. DEAE-cellulose (DE-52, pre-swollen) was purchased from Reeve Angel, Clifton, N.J., U.S.A. Sephadex G-75 was from Pharmacia, Montreal, Que., Canada and L-[³H]serine (specific radioactivity 60 mCi/mg) was from Amersham/Searle, Toronto, Ont., Canada. [³H]Phenylalanine (specific radioactivity 100 mCi/mg) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. Acrylamide and bisacrylamide were products of Eastman Organic Chemicals, Rochester, N.Y., U.S.A. PCS scintillation solution was obtained from Amersham/Searle.

Results

Control experiments

As a number of factors, such as non-specific binding of aminoacyl-tRNA to sites on polyribosomes other than the A or P site, or non-specific binding of amino acid to polyribosomes, could affect the specific radioactivity of the amino acid isolated from polyribosome aminoacyl-tRNA, control experiments were performed to determine whether or not these factors affected the results. The results in Table 1 show, first, that approx. 5 and 7% of [³H]seryl-tRNA and [³H]phenylalanyl-tRNA respectively co-sediment with polyribosomes on centrifugation of muscle S-10 supernatant mixed with the tRNA species. Secondly, the amount of tRNA isolated from polyribosomes is similar to the quantity normally isolated in the absence of added tRNA. Since each ribosome contains one molecule of 5S RNA (Knight & Darnell, 1967; Nomura, 1970) the ratio of tRNA/5S RNA would give the number of tRNA molecules bound per ribosome. The results in Table 1 show that the tRNA/5S RNA ratio for the polyribosome preparation is approx. 2, suggesting that non-specific binding of tRNA to sites on polyribosomes other than the A and P sites does not occur, as each ribosome is expected to carry two tRNA molecules (Warner & Rich, 1964; Wettstein & Noll, 1965).

A possible source of error in the experiments is the sedimentation with the polyribosomes of complexes of aminoacyl-tRNA synthetases and aminoacyl-tRNA, which occur in other systems (Bandyopadhyay & Deutscher, 1971; Vennegoor *et al.*, 1972). The effect of such co-sedimentation would be to contaminate polyribosome aminoacyl-tRNA with aminoacyl-tRNA from another source. The results of experiments with polyribosomes as source of aminoacyl-tRNA synthetase showed that the maxi-

mum amino acid acceptance (with 1 mg of ribosomal protein) is about 6.5% of the specific serine- or phenylalanine-accepting activity of muscle homogenate. This result suggests that under the experimental conditions only a negligible quantity of aminoacyl-tRNA synthetases co-sediment with polyribosomes.

Another possible source of error is the binding of amino acid to tRNA resulting in co-elution of [³H]serine or [³H]phenylalanine with tRNA during chromatography on DEAE-cellulose or Sephadex. Control experiments were therefore performed to determine the extent of this co-elution. After extensive washing of the DEAE-cellulose column with at least 250 ml of 0.1M-sodium acetate, pH 4.4, very little [³H]serine or [³H]phenylalanine (which is calculated to be about 0.5% of the applied radioactivity) was eluted with the tRNA. After chromatography of RNA on Sephadex G-75 columns, no radioactivity was eluted with the tRNA peak, eliminating the possibility that free amino acids contaminate polyribosome aminoacyl-tRNA.

Sodium dodecyl sulphate was included in the homogenization buffer to eliminate ribonuclease degradation of aminoacyl-tRNA. The results in Table 2 show that degradation of [³H]phenylalanyl-tRNA and [³H]seryl-tRNA by the polyribosome fraction is approx. 13–14% higher than the degradation in the absence of polyribosomes.

This suggests that the experimental procedure protects the aminoacyl-tRNA from significant breakdown.

Specific radioactivity of polyribosome aminoacyl-tRNA

The kinetics of acylation *in vivo* were studied with aminoacyl-tRNA purified by chromatography on DEAE-cellulose and Sephadex. The RNA profiles

Table 1. Binding of [³H]seryl-tRNA and [³H]phenylalanyl-tRNA to polyribosomes

[³H]Phenylalanyl-tRNA (11400 c.p.m.) or [³H]seryl-tRNA (8700 c.p.m.) prepared from leg muscle was incubated with S-10 supernatant (2.5 mg of protein) for 30 min at 4°C and the mixture was centrifuged at 200000g for 1 h. The radioactivity content of the polyribosome pellet and the S-200 supernatant was determined. In the 'no addition' experiment, muscle S-10 supernatant (2.5 mg of protein) was centrifuged at 200000g for 1 h and the tRNA/5S RNA ratio was determined: the radioactivity detected represents blank value. tRNA and 5S RNA were prepared from the polyribosome pellet by phenol extraction and separation on a Sephadex G-75 column (Nwagwu & Nana, 1974).

Expt.	Radioactivity in polyribosome pellet (c.p.m.)	Radioactivity in S-200 fraction (c.p.m.)	Polyribosome tRNA (E_{260} unit/2.5 mg of protein in S-10 supernatant)	tRNA/5S RNA
S-10 (No addition)	25	28	0.24	1.99
S-10 + [³ H]seryl-tRNA (5 E_{260} units; 8700 c.p.m.)	480 (5.52)*	7875	0.22	1.87
S-10 + [³ H]phenylalanyl-tRNA (5 E_{260} units; 11400 c.p.m.)	800 (7.12)*	10150	0.23	1.95

* Percentage of total radioactivity that co-sediments with polyribosomes.

Table 2. Degradation of [^3H]phenylalanyl-tRNA by the polyribosomal fraction

Two 5g portions of 14-day embryo muscle were homogenized in 0.3M-NaCl-0.01M-MgCl₂-0.01M-Tris-HCl, pH6.8, and centrifuged at 10000g at 2°C for 10min. The resulting supernatant was centrifuged at 200000g for 1h at 2°C. To the polyribosome pellet of sample 1, 41 500c.p.m. of [^3H]phenylalanyl-tRNA was added simultaneously with resuspension of the pellet in 4ml of 1% sodium dodecyl sulphate-0.02M-EDTA-0.02M-sodium acetate, pH4.4. The tubes were thoroughly mixed. To the polyribosome fraction of sample 2, 83 500c.p.m. of [^3H]phenylalanyl-tRNA was similarly added. A tube containing 41 700c.p.m. of [^3H]phenylalanyl-tRNA (20 E_{260} units) and 1% sodium dodecyl sulphate-0.02M-EDTA-0.02M-sodium acetate, pH4.4, was included as control. RNA was extracted from the samples and purified by DEAE-cellulose chromatography as described in the Methods section. Radioactivity contained in the RNA peak was determined.

Sample	[^3H]Phenylalanyl-tRNA added (c.p.m.)	[^3H]Phenylalanyl-tRNA recovered (c.p.m.)	Recovery (%)
Control	41 700	35 650	85.5
Sample 1	41 500	30 100	72.5
Sample 2	83 500	59 500	71.2

Fig. 1. Sephadex G-75 chromatography of polyribosome [^3H]phenylalanyl-tRNA labelled *in vivo*

[^3H]Phenylalanyl-tRNA was prepared after labelling for 0.5 min (a) or 5.5 min (b). Tubes 48–53 of both (a) and (b) were pooled as [^3H]phenylalanyl-tRNA. The chromatographic profiles for the samples incubated for 2.5 min are omitted since they are similar to those for 5.5 min. The first E_{260} peak is rRNA; the second peak is 5S RNA and the third, tRNA. The profiles for [^3H]seryl-tRNA are identical with those presented for [^3H]phenylalanyl-tRNA and are therefore not shown. ●, Radioactivity; —, E_{260} .

after Sephadex chromatography are shown in Fig. 1. The results of preliminary experiments showed that [^3H]phenylalanyl-tRNA and [^3H]seryl-tRNA, prepared *in vitro* with embryonic muscle tRNA and synthetase, was each eluted in tubes 45–55 on chromatography on a Sephadex G-75 column. Peptidyl-tRNA, depending on its size, was eluted in tubes 32–45. Accordingly, the fractions that eluted in tubes 48–53 (Figs. 1a and 1b) after chromatography of polyribosome aminoacyl-tRNA species labelled for 0.5, 2.5 and 5.5 min respectively were separately pooled and used as aminoacyl-tRNA. No radioactivity was eluted in the position expected for amino acids on Sephadex chromatography of both poly-

ribosome aminoacyl-tRNA species, eliminating the possibility of degradation of aminoacyl-tRNA on Sephadex columns.

The result presented in Fig. 2 shows that the specific radioactivity of phenylalanyl-tRNA and seryl-tRNA increases rapidly up to 30s, after which it increases gradually up to 2.5 min and then reaches a plateau. However, in the absence of any time-points between 30s and 2.5 min it is possible that the plateau is reached earlier than 2.5 min. At the plateau of aminoacylation, *in vivo*, the maximum efficiency of serine acceptance by polyribosome tRNA is 20 pmol of serine/ E_{260} unit of tRNA; the efficiency of phenylalanine acceptance is 30 pmol/ E_{260} unit of tRNA (Fig. 2).

Fig. 2. Kinetics of polyribosome phenylalanyl-tRNA and seryl-tRNA formation *in vivo*

[³H]Seryl-tRNA (○) and [³H]phenylalanyl-tRNA (△) were extracted from the polyribosome fraction and purified by Sephadex G-75 chromatography, as shown in Fig. 1. Tubes 48–53 of Figs. 1(a) and 1(b) were pooled as aminoacyl-tRNA. For the 2.5 min acylation time (not shown in Fig. 1), fractions corresponding to those of Figs. 1(a) and 1(b) were pooled as aminoacyl-tRNA. The radioactivity of aminoacyl-tRNA was corrected for the radioactivity in the S-10 supernatant fraction that is soluble in cold trichloroacetic acid (given as μg of serine). Specific radioactivity is measured as c.p.m. of [³H]phenylalanyl-tRNA $\cdot E_{260} \text{ unit}^{-1} \cdot \mu\text{g}$ of [³H]phenylalanine⁻¹ and c.p.m. of [³H]seryl-tRNA $\cdot E_{260} \text{ unit}^{-1} \cdot \mu\text{g}$ of serine⁻¹. Each point on the graph represents an average of three separate experiments. Specific serine (●) and phenylalanine (▲) acceptance was determined as described by Nwagwu & Lianga (1974).

Discussion

The results of this study show that radioactively labelled aminoacyl-tRNA essentially free from contamination can be prepared from muscle polyribosomes.

The validity of our results depends to a great extent on the purity of the tRNA and polyribosomes. tRNA was extensively purified by chromatography on both DEAE-cellulose and Sephadex G-75. These procedures give tRNA which migrates as a single peak of 4S RNA on polyacrylamide-gel electrophoresis and which has a high activity in amino acid acceptance (A. Hossain, unpublished work).

The results of control experiments make it highly unlikely that (i) aminoacyl-tRNA binds non-specifically to sites on polyribosomes other than the A and P sites; (ii) significant amounts of aminoacyl-tRNA synthetase tRNA complexes co-sediment with polyribosomes; (iii) [³H]serine or [³H]phenylalanine are eluted together with tRNA during chromatography on DEAE-cellulose, or Sephadex G-75 and (iv) aminoacyl-tRNA is significantly degraded during the experiment.

Since polyribosomal aminoacyl-tRNA is the immediate precursor for polyribosome peptides, its specific radioactivity would be expected rapidly to reach a maximum value which may be maintained throughout protein synthesis, as long as amino acids are readily available. The specific radioactivity of polyribosome peptides, namely myosin, actin and tropomyosin, reaches a maximum within an average of 10 min (Herrmann *et al.*, 1971). As would be expected of a precursor-product relationship, the specific radioactivity of polyribosomal aminoacyl-tRNA reaches a maximum well ahead of polyribosome peptides.

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