Studies on the Fractionation of Total Transfer Ribonucleic Acid and Purification of an Alanine Transfer Ribonucleic Acid from Chick Embryo

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Chick embryo tRNA, prepared by a simple large-scale method, was fractionated on three different ion-exchange columns. In all cases simple chromatographic patterns for various tRNA species were observed, indicating the presence of only a few major species of tRNA for each amino acid. By repeated chromatography one species of alanine tRNA was purified to approx. 80% purity. T₁ ribonuclease digest of this purified tRNA gave a simple chromatographic pattern. Because of the simplicity of the method of preparation of tRNA from this readily available source and the presence of only a few species of tRNA for each amino acid, chick embryo is suited for the study of tRNA and its various functions in higher systems.

Some of the present day interests in tRNA are in the study of its primary sequence from diverse sources, X-ray crystallography to gain an insight into the three-dimensional structure and study of the structure-function relations to locate the active sites on the tRNA molecule involved in many of its biological interactions. tRNA has been implicated in cellular processes such as viral infection (Sueoka & Kano-Sueoka, 1964), sporulation (Kaneko & Doi, 1966), neoplastic changes (Holland *et al.*, 1967) and differentiation and development (Yang & Comb, 1968); this latter aspect has added a new dimension to the interest in the study of these molecules.

More than 40 tRNA species have been sequenced, most of them from micro-organisms, especially yeast and *Escherichia coli*. Only three of them are from higher systems, i.e. the phenylalanine tRNA from wheat germ (Dudock *et al.*, 1969), serine tRNA from rat liver (Staehelin *et al.*, 1968) and phenylalanine tRNA from rabbit liver (Keith *et al.*, 1973). The main difficulties in the determination of the structure of tRNA from higher systems are in obtaining large quantities of fresh starting material for bulk preparation of tRNA and in removing DNA which requires elaborate procedures, especially from mammalian sources. Because of the difficulty in getting highly labelled tRNA from higher systems, the method of Sanger *et al.* (1965) has only limited applications in such cases. Though for sequence studies small quantities of highly labelled tRNA may be used, for most other studies, substantial amounts of purified tRNA species are required.

A method for large-scale preparation of crude calf liver tRNA and the purification therefrom of five specific tRNA species has been described (Pearson

et al., 1973). We have now developed a simple method for the large-scale preparation of total tRNA from whole chick embryo (Anandaraj & Cherayil, 1974) and we report here the chromatographic fractionation of chick embryo tRNA and isolation of alanine tRNA of approx. 80% purity with an overall yield of 25% of the total alanine acceptor activity.

Materials and Methods

Incubated eggs were from a pure strain of white Leghorn hens in the Government Poultry Farm, Hassaraghetta, Bangalore, India. Phenol from Sarabhai Merck (Baroda, India) was melted with 20% (v/w) water and used without purification. Radiochemicals were obtained either from The Radiochemical Centre, Amersham, Bucks., U.K. (Am), or Bhabha Atomic Research Centre, Bombay, India (BARC). The specific radioactivities of these in mCi/mmol were as follows: [¹⁴C]alanine (BARC), 33; [¹⁴C]aspartic acid (BARC), 25; [¹⁴C]histidine (Am), 150; [¹⁴C]serine (Am), 42; [¹⁴C]valine (Am), 125; [³H]proline (Am), 6800; [³H]lysine (Am) 2500; [³H]isoleucine (Am), 15000. [¹⁴C]Tyrosine was a gift from Professor H. G. Khorana (Department of Biology & Chemistry, MIT, Mass., U.S.A.) and its specific radioactivity is not known. BD-cellulose (20–50 mesh) was from Schwarz Bioresearch Inc., Orangeburg, N.Y., U.S.A. DEAE-cellulose (0.5 mequiv./g) was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. DEAE-Sephadex A50 was from Pharmacia Fine Chemicals, Uppsala, Sweden. T₁ Ribonuclease was obtained from Calbiochem, Los Angeles, Calif., U.S.A.

Methods

Preparation of tRNA. tRNA was prepared by the simple method developed in this laboratory. In this method, 15-day-old live embryos were homogenized in sodium acetate buffer (pH 4.5) containing 1% sodium dodecyl sulphate and NaCl (6ml/g of tissue), the final concentration of Na^+ being 0.14M. Some 500 embryos (weighing approx. 5kg) were processed at a time. The homogenate was distributed between two polyethylene vessels and stirred continuously for 1h with 12 litres of liquefied phenol. After 40h of settling under gravity, the aqueous phase was siphoned out and further deproteinized with phenol twice more. The deproteinized aqueous phase was directly passed through DEAE-cellulose previously equilibrated with 0.14M-NaCl. The column was washed with 0.14M-NaCl, the tRNA was eluted with 1M-NaCl and precipitated with 2.5vol. of 95% ethanol. The precipitate was collected by centrifugation, washed twice with 95% ethanol and dried under vacuum over CaCl_2 . All these operations were carried out at room temperature. The yield of tRNA was 0.4g/kg of tissue.

Column chromatographic procedure. Fractionation of tRNA was carried out on three different ion-exchange columns, i.e. DEAE-cellulose, DEAE-Sephadex and BD-cellulose. DEAE-cellulose chromatography was done under two conditions, one at pH 7.2 and the other at pH 4.5, with an exponential gradient of urea with a concomitant shallow gradient of NaCl (Cherayil & Bock, 1965). BD cellulose chromatography was done by the method of Gillam *et al.* (1967) with a linear gradient of NaCl 0.45–1M in the presence of 0.05M-sodium acetate, pH 4, and 0.01M- MgCl_2 . DEAE-Sephadex chromatography was done in the presence of 7M-urea with a linear gradient of NaCl, pH 7.6 (Bock & Cherayil, 1967).

Preparation of fractions for assay. Suitable portions of the chromatographic fractions were plated on Whatman no. 3 paper discs (1.5cm $\times$ 1.5cm) and dried. Urea and salts were removed by washing with aq. 75% (v/v) ethanol containing 0.05M-KCl. The tRNA on the paper was assayed by the method of Cherayil & Bock (1965).

Preparation of aminoacyl-tRNA synthetase. Embryos (11 days old) were homogenized in a buffer (buffer A) containing 10mM-potassium maleate (pH 7.2), 10mM- MgCl_2 , 30mM- NH_4Cl and 5mM-2-mercaptoethanol; 2.5ml of buffer A was used/g wet wt. of tissue. The homogenate was centrifuged at 25000g in a Sorvall refrigerated centrifuge for 10min. The supernatant liquid was further centrifuged at 105000g in a Spinco (model L) preparative ultracentrifuge for 1h. The supernatant fluid thus obtained was freed of endogenous tRNA and amino acids by DEAE-cellulose chromatography (Muench & Berg, 1966). For this the supernatant liquid was

diluted with an equal volume of 5mM-2-mercaptoethanol to decrease the salt concentration. Diluted supernatant fluid (30ml) was adsorbed on a DEAE-cellulose column (2cm $\times$ 10cm) equilibrated with buffer A diluted to half its molarity. The column was washed with 60–80ml of diluted buffer A and the aminoacyl-tRNA synthetases were eluted with buffer A containing 0.3M-KCl. Fractions of volume 2.5ml were collected. Fractions with absorbance above 0.2 at 280nm were pooled and used as the synthetase.

Amino acid acceptor assay. The reaction mixture contained in a volume of 0.15ml; 50mM-potassium maleate (pH 7.2); 5mM- MgCl_2 ; 0.5mM-EDTA; 5mM-2-mercaptoethanol; 1mM-ATP; 0.5mM-CTP; 0.1 μCi of ^{14}C -labelled amino acid or 1 μCi of ^3H -labelled amino acid; enzyme protein, approx. 300–350 μg . This reaction mixture was applied to the paper discs containing the tRNA. Incubation was done in a moist chamber for 30min, at the end of which the paper discs were partially dried in a current of hot air and put into 10% (w/v) trichloroacetic acid containing unlabelled amino acids. The paper discs were sandwiched between two filter papers and washed in a Büchner funnel several times with 5% trichloroacetic acid followed by ether-ethanol mixture (1:3, v/v), dried and counted for radioactivity in a liquid-scintillation counter (Beckman LS100) with 0.5% 2,5-diphenyloxazole in toluene as the scintillation fluid.

T_1 Ribonuclease digestion. The purified tRNA was acylated with [^{14}C]alanine by appropriately scaling up the incubation components. After incubation the tRNA was deproteinized with phenol saturated with acetate buffer, pH 4.5, and recovered by adsorption to DEAE-cellulose followed by elution with 1M-NaCl and precipitation with ethanol. It was then digested with T_1 ribonuclease in the presence of 5mM-EDTA and 10mM-sodium acetate (pH 5.4) at 37°C for 12h (Smith *et al.*, 1966). For every 20 E_{260} units of tRNA 30 units of enzyme were used. At the end of the incubation the digest was made 3M with respect to urea by the addition of solid urea and was added to a column (0.8cm $\times$ 40cm) of DEAE-cellulose. The column was developed with a linear gradient of NaCl (0–0.4M) in a total volume of 500ml, in the presence of 3M-urea and 0.02M-sodium acetate (pH 4). Fractions of volume 1.2ml were collected. Samples (0.1ml) were plated on Whatman no. 3 filter-paper discs, dried and counted for radioactivity as above.

Results

A preliminary chromatography carried out with 1.5g of total tRNA on a DEAE-cellulose column (pH 7.6) resulted in the partial separation of different species of tRNA. Of the chromatographic patterns studied for the tRNA species for 16 amino acids, 10 are represented in Figs. 1(a), 1(b) and 1(c). Aspartic acid tRNA was eluted in the front as a peak with

Fig. 1. Elution profiles of tRNA from different columns

(a), (b) and (c) DEAE-cellulose chromatography (pH 7.2) was done on a column (5.1 cm x 30 cm) with an exponential gradient of urea (1.5M-6M) with 0.35-0.42M NaCl, in the presence of 0.01 M-Tris-HCl buffer. The constant-volume mixer contained 1.2 litres. The flow rate was 200 ml/h and fractions (12 ml) were collected. (a) Serine; O, aspartic acid; □, valine; ■, leucine. (b) Alanine; □, histidine; O, proline. (c) Tyrosine; O, isoleucine; □, lysine. (d) and (e) DEAE-cellulose chromatography (pH 4.5) was done on a column (2 cm x 60 cm) with an exponential gradient of urea (1.5-7M) with 0.02M sodium acetate. The constant-volume mixer contained 200 ml. Flow rate was 27 ml/h and fractions (7.5 ml) were collected. (d) ●, Aspartic acid; △, valine; □, tyrosine. (e) ●, Alanine; O, proline; □, histidine. (f) BD-cellulose chromatography was done on a column (0.9 cm x 35 cm) at a flow rate of 12 ml/h. Fractions of volume 5.5 ml were collected. Total gradient was 400 ml. Further details are given under 'Methods'. (f) □, Lysine; ●, proline; O, histidine. —, E₂₆₀.

a shoulder (Fig. 1a). Proline tRNA was separated into two well-defined peaks, the second peak having a shoulder (Fig. 1b). The profiles for histidine and alanine tRNA species suggested the presence of two species of tRNA for each amino acid (Fig. 1b). Tyrosine tRNA was eluted as a single asymmetric peak which suggested the presence of more than one species (Fig. 1c). Valine tRNA was separated into two major peaks (Fig. 1a). Leucine and serine tRNA profiles showed the presence of multiple species for each amino acid (Fig. 1a). The presence of at least four species for leucine was also evident from DEAE-Sephadex column chromatography. Similar simple patterns were noted for tRNA species for other amino acids as well. The chromatographic fractions were pooled into five lots and these were rechromatographed either on DEAE-cellulose, pH 4.5, or BD cellulose, pH 4.

When the pooled fractions 20–30 (Fig. 1, lot 1) were rechromatographed on the DEAE-cellulose column (pH 4.5), the partially separated aspartic acid tRNA was resolved into two well-defined peaks (Fig. 1d). The same lot containing one of the species of valine was not resolved any further, whereas tyrosine tRNA was resolved into two peaks (Fig. 1d).

Histidine acceptor peak, which indicated the presence of two species, did not resolve further on rechromatography of lot (2) on a DEAE-cellulose column (pH 4.5) or lot (4) on a BD-cellulose column (Figs. 1e and 1f). In almost all cases simple patterns similar to that shown in Fig. 1 were observed on rechromatography on DEAE-cellulose (pH 4.5) or BD-cellulose (pH 4) columns. But, in the case of asparagine tRNA, rechromatography of a fraction resulted in multiple peaks (not shown in figure).

One of the alanine peaks (hereafter referred to as $\text{tRNA}_1^{\text{Ala}}$) (Fig. 1e) was further chromatographed on two more columns (BD-cellulose and DEAE-Sephadex) and one fraction rich in alanine acceptor activity was obtained. The entire fractionation procedure for the purification of $\text{tRNA}_1^{\text{Ala}}$ was repeated starting with 2.5 g of total tRNA (Fig. 2). In the first DEAE-cellulose column chromatography the two alanine tRNA species separated well (Fig. 2a). Fractions 40–60 containing the major species were pooled and half of it was rechromatographed on a DEAE-cellulose column (pH 4.5). There was partial separation of absorbance and the alanine tRNA was eluted as a single peak (Fig. 2b). Fractions 47–60 were then pooled and rechromatographed on a BD cellulose column when there was distinct separation of the isospecies, each eluting out as a symmetrical peak (Fig. 2c). When fractions 48–64 from the BD-cellulose column were chromatographed on a DEAE-Sephadex column, the alanine-accepting fraction was eluted as a single peak (Fig. 2d). However, the acceptor activity did not perfectly coincide with the absorbance peak, indicating some contamination.

Fig. 2. Profiles of alanine tRNA in different columns

(a) DEAE-cellulose column (pH 7.2). Details are as in Fig. 1. (b) DEAE-cellulose column (pH 4.5). All the details are as in Fig. 1(d), except that the constant-volume mixer contained 400 ml and the fraction volume was 4.2 ml. (c) BD-cellulose chromatography was done on a column (1.2 cm $\times$ 40 cm) with a total gradient of 600 ml at a flow rate of 30 ml/h. Fractions of volume 4.2 ml were collected. For details of the gradient see under 'Methods'. (d) DEAE-Sephadex chromatography was done on a column (0.9 cm $\times$ 40 cm) at a flow rate of 8.6 ml/h. A linear gradient of 0.46–0.63 M-NaCl in the presence of 0.02 M-Tris and 7 M-urea was used. Total gradient was 150 ml. —, E_{260} ; $\circ$, radioactivity.

Table 1. Purification of alanine $\text{tRNA}_1^{\text{Ala}}$

The molecular weight of tRNA was assumed to be 30000, and 1 mg of the tRNA corresponded to 22 E_{260} units. Only half of the tRNA recovered from the first DEAE-cellulose column 3 was loaded on the second and subsequent columns.

Method	Recovery (E_{260} units)	Specific activity (nmol of alanine/mg of tRNA)
Crude	50910	0.84
DEAE-cellulose (pH 7.2)	10790	2.03
DEAE-cellulose (pH 4.5)	3120	4.90
BD-cellulose	720	7.00
DEAE-Sephadex	330	25.00

Fig. 3. DEAE-cellulose chromatography of T_1 ribonuclease digest of (a) purified tRNA^{Ala} and (b) total chick embryo tRNA. The details of chromatography are given under 'Methods'. $\blacktriangle$, Radioactivity; $\circ$, E_{260} .

Fractions 41–69 amounted to 8 mg of tRNA which accepted 25 nmol of alanine/mg. This sample when assayed with ^{14}C -labelled algal protein hydrolysate containing excess of unlabelled alanine, indicated the presence of less than 20% of contaminating tRNA species. The details of the recovery and percentage enrichment of the alanine tRNA at various stages are given in Table 1. The net recovery of tRNA^{Ala} was 25%.

The purified sample was acylated with [^{14}C]-alanine, digested with T_1 ribonuclease at pH 5.4 (to prevent deacylation of the tRNA during digestion)

and chromatographed on a DEAE-cellulose column at pH 4.0. The chromatographic profile is shown in Fig. 3(a). Twelve fairly well separated peaks were observed, of which the second and sixth peaks were partially resolved into two peaks each. The whole of the radioactivity representing the 3' end of the tRNA molecule was eluted as a single peak with a small shoulder. Total tRNA, when digested with T_1 ribonuclease and chromatographed in a similar way, gave a pattern shown in Fig. 3(b). In general, particularly between fractions 140–160, separation of the oligonucleotides into distinct peaks was not

as well-defined as in Fig. 3(a). This is presumably indicative of a large number of different oligonucleotides in the total tRNA digest.

Discussion

In the study of multicellular systems it is the practice to use individual organs or a single type of cells for biochemical investigations; the organism as a whole is rarely used. In the present studies whole embryos were used as it was convenient. Moreover, the tRNA of a whole live embryo is less susceptible to nuclease action than that of an isolated organ. As the main role of tRNA species in all the organs of an animal is in protein synthesis, it is reasonable to expect only a few tRNA species for each amino acid, organ-specific tRNA species being rare. In agreement with this is the work of Taylor *et al.* (1968) who studied tRNA species from various organs of different animals and observed no appreciable chromatographic differences in the number and types of tRNA species in them. White *et al.* (1973) found only a few major isoaccepting species of tRNA for a number of amino acids in the whole *Drosophila* at various stages of development, although a number of minor isoaccepting species were seen at certain stages.

In the present studies we find in general a limited number of isoaccepting species under three different column chromatographic conditions; very few minor species of tRNA could be detected in any of the chromatographic procedures. The fact that there are only a few major species of tRNA in chick embryo enhances the possibility of using this system for the purification of specific tRNA species. This has been demonstrated by the purification of one species of alanine tRNA, by using four standard column chromatographic procedures.

The amount of alanine tRNA present in the starting material was 2.5–3%. The first two columns both gave a 2.4-fold purification, whereas the third column gave only 1.4-fold purification over the previous one. The last column gave 3.6-fold purification. Even after four chromatographic runs the recovery was 25% on the basis of both the isoacceptors of alanine being present in the total tRNA. The amino acid acceptance of the purified tRNA corresponds to 75% purity. Since the acceptance of other amino acids by the purified material was less than 20% we believe that the tRNA was at least 80% pure.

In the T_1 ribonuclease digest of unfractionated tRNA, a large number of oligonucleotides, derived from the various tRNA species will be present and hence it is difficult to separate these chromatographically into well-defined absorbance peaks (Fig. 3b). In the case of a purified sample containing a homogeneous population of molecules only a limited number of oligonucleotides are expected and therefore separation will be better (Reeves *et al.*, 1968). This

limited number of peaks of oligonucleotides obtained from the purified tRNA^{Ala} (Fig. 3a) suggests that the sample contained a fairly homogeneous population of tRNA molecules. The contribution to the absorbance from the contaminating tRNA species is not more than 20%, as the acceptance for alanine has been found to be about 80%. It is quite unlikely that the contaminant is a single tRNA since we have noted the presence, in small amounts, of a few other tRNA species in the tRNA^{Ala} fraction (M. P. J. S. Anandaraj & J. D. Cherayil, unpublished work). As the sequences of the contaminating tRNA species cannot be identical, the oligonucleotides produced will be different and hence each will be present in small amounts. Hence these oligonucleotides should not show up as large absorbance peaks. The peaks noted therefore can be expected to be mostly from the major species, namely tRNA^{Ala}. The pattern obtained by chromatography of the T_1 ribonuclease digest thus provides supporting evidence to the observation on the purity (about 80%) of the tRNA^{Ala}, on the basis of its acceptance of amino acid. The shoulder observed in the peak of the radioactivity probably indicates the presence of a small amount of an unresolved isoacceptor which is very much similar to tRNA^{Ala}.

The procedure for preparing bulk quantities of tRNA has been worked out for processing 5 kg of the tissue at a time, although there is no difficulty in scaling it up to any desired amount, provided adequate facilities are available. The tRNA yield of 0.4 mg/g of tissue is comparable with that obtained from other animal tissues (Pearson *et al.*, 1973; Brungraber, 1962). Since it was found that the tRNA content/g of tissue was highest on day 15 of development (Maya Sundari, 1972), embryos at this stage were used for preparation of tRNA. The availability of pure strains of chick embryos, ease of preparation of total tRNA in bulk and the presence of a limited number of isoaccepting species of tRNA, make chick embryo a good system for purification in bulk of specific tRNA by using standard column chromatographic procedures.

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