Extensive Charging of Transfer Ribonucleic Acid by Bean Leaf Extracts *in vitro*

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1. Phenol was effectively removed from aqueous extracts of RNA by chromatography on Sephadex G-50. 2. Elution of tRNA from Sephadex G-50 columns at pH 7.6 was shown to remove 91% of the endogenously bound amino acids. 3. tRNA prepared without recourse to ethanolic precipitation was capable of accepting much greater amounts of amino acids than could redissolved samples of precipitated tRNA. 4. Aminoacyl-tRNA synthetase enzymes were partially purified with calcium phosphate gel. Elution of enzymes from the gel at pH 6.5 yielded a fraction having phenylalanine- and alanine-charging activity, but no aspartate-, lysine- or proline-charging activity, whereas elution at pH 7.6 gave a fraction having aspartate-, lysine- and proline-charging activity but no phenylalanine- or alanine-charging activity. 5. By using partially purified synthetase enzymes and tRNA eluted from DEAE-Sephadex A-50 columns, 52% of the theoretical maximum of aminoacyl-tRNA synthesis was obtained *in vitro*.

Regulation of protein synthesis at the level of translation has been discussed by several authors (Stent, 1964; Armstrong, 1966; Gross, 1968; Korner, 1970; Sueoka & Kano-Sueoka, 1970). The formation of aminoacyl-tRNA complex has been postulated as a critical step in translational regulation of protein synthesis in plants (Vold & Sypherd, 1968; Anderson & Cherry, 1969; Vanderhoef & Key, 1970; Tao & Hall, 1971).

To study the role of aminoacyl-tRNA formation as a regulatory step in protein synthesis it is essential that extracts giving high rates of complex formation *in vitro* be obtained. A previous study (Hall & Tao, 1970) revealed that the rates of aminoacyl-tRNA synthesis by bean leaf preparations *in vitro* were very low compared with the rates obtained *in vivo*. Optimization of reaction conditions (Tao & Hall, 1971) resulted in a 24-fold increase in reaction rate *in vitro*. However, endogenous sources of amino acids still prevented the realization of a high apparent rate of aminoacyl-tRNA formation and protein synthesis (Hall & Tao, 1970; Sela, 1969). It was evident that further purification of the enzyme and tRNA fractions was necessary before rates *in vitro* approaching those *in vivo* would be obtained. The present paper describes techniques that effectively removed endogenous amino acids from tRNA and resulted in rapid and extensive charging *in vitro* of supplied tRNA.

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EXPERIMENTAL

Materials. Rapidly expanding leaves of *Phaseolus vulgaris* L. (var. Tendergreen) plants grown under artificial illumination (3500 ft-candles) with 16h days (26°C day, 17°C night) in the University of Wisconsin Biotron were used in all experiments. Small (8 mm × 200 mm) plastic Chromaflex columns used to separate the products of reactions *in vitro* were obtained from Kontes Glass Co., Vineland, N.J., U.S.A. Hepes [1-(N-2-hydroxyethyl)piperazine-N'-yl]ethanesulphonic acid], diethiothreitol and Aquacide II (a dehydrating agent) were the products of Calbiochem, Los Angeles, Calif., U.S.A. Polyvinylpyrrolidone was obtained as the insoluble white powder 'Polyclar AT' from General Aniline and Film Corp., Grasselli, N.J., U.S.A. Trizma 7.0 was a co-crystallized compound of tris and tris-HCl, giving a pH value of 7.5 at 5°C, purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Radioactive amino acids were obtained from Amersham/Searle, Des Plaines, Ill., U.S.A. The ¹⁴C-labelled amino acid mixture used had a specific radioactivity of 54 mCi/mg-atom of C and the same composition as given previously (Tao & Hall, 1971). Individual L-amino acids were: [¹⁴C]alanine (105 mCi/mmol); [³H]-aspartic acid (385 mCi/mmol); [³H]lysine (256 mCi/mmol); [³H]proline (205 mCi/mmol); [³H]phenylalanine (750 mCi/mmol).

Preparation of tRNA-rich RNA. Four methods were employed to obtain RNA with amino acid-acceptor activity. (1) Ethanol precipitation. RNA was extracted with a phenol-buffer system and concentrated by ethanol precipitation as described by Tao & Hall (1971). (2) Sephadex G-50 chromatography. Total RNA was extracted with the phenol-buffer system and the crude extract centrifuged at 14000g_{av} for 10 min. The aqueous layer was pipetted off and extracted with an equal

volume of phenol. This was repeated, then the aqueous layer was added to a Sephadex G-50 column (1.5 cm $\times$ 70 cm) equilibrated with 20 mM-tris-HCl buffer, pH 7.5, containing 375 mM-NaCl and 8 mM-MgCl₂. RNA was eluted from the column with the same tris buffer and 100-drop fractions were collected. The column effluent was monitored at 260 nm with a 1 cm flow cell in a Gilford 2000 spectrophotometer system. These phenol-free total cellular RNA fractions were pooled together and used directly, or further purified by procedure (3) or (4). (3) Sodium acetate fractionation. The RNA eluted from the Sephadex G-50 column was adjusted to 3 M-sodium acetate and left at 4°C for 12 h (Vanderhoef, Bohannon & Key, 1970). High-molecular-weight RNA precipitated and was sedimented by centrifugation. The supernatant was dialysed in the cold against three changes of 8 mM-MgCl₂ adjusted to pH 7.5 with a small amount of Trizma 7.0 buffer crystals, then concentrated by covering the dialysis bag with Aquacide II for about 2 h. (4) DEAE-Sephadex A-50 column chromatography. The procedures of Moriyama, Ip & Busch (1970) for column packing were followed. The column (2.5 cm $\times$ 40 cm) was equilibrated with the starting buffer and RNA (15 mg) obtained by method (2) above was added to the column. The RNA was eluted with a linear gradient from 375 mM-NaCl to 1 M-NaCl. Both the starting buffer (400 ml) and the high-salt buffer (400 ml) contained 8 mM-MgCl₂ in 20 mM-tris-HCl buffer, pH 7.5. Fractions (100 drops) were collected at a flow rate of 12 drops/min. The column effluent was monitored at 260 nm as stated above. The pooled fractions of each peak were concentrated with Aquacide.

Preparation of aminoacyl-tRNA synthetases. Freshly collected leaves (50 g) were cut into and then ground within 90 s in 200 ml of extraction buffer containing: 0.1 M-Hepes-KOH, pH 7.4, 5 mM-dithiothreitol, 1 mM-GSH, 20 g of polyvinylpyrrolidone and 10 g of Dowex 50 (X8; Na⁺ form). The grinding flask was surrounded by crushed ice and the contents were continually flushed with N₂ supplied through a side arm. The extract was filtered with squeezing (by using gloves) through acetate taffeta cloth into a cold beaker flushed with N₂. This initial filtrate was immediately re-filtered under suction through a Büchner funnel containing 6 g of Sephadex G-50 (previously swollen in extraction buffer) which rested on two layers of Miracloth (Chicopee Mfg. Co., Milltown, N.J., U.S.A.) and which was covered with a glass-wool pad. The filtrate was collected in a Büchner flask surrounded by crushed ice and containing 10 g (635 mg dry wt.) of calcium phosphate gel in 0.1 M-Hepes-KOH, pH 7.4–5 mM-dithiothreitol–1 mM-GSH. The top of the funnel was flushed with N₂ and the system stood on a magnetic stirrer so that the filtrate was continually stirred under N₂ into the buffered slurry of calcium phosphate gel. When filtration was complete the Büchner flask was stoppered and placed in a cold-room at 4°C where the extract was stirred for a further 10 min. The procedure for elution of synthetase enzyme fractions from the gel is presented in Scheme 1.

In some experiments a pH 5 enzyme fraction was used. This was prepared as described by Hall & Tao (1970).

Assay for aminoacyl-tRNA synthesis. The filter-paper disc method of Rubin, Kelmers & Goldstein (1967) was used as described by Tao & Hall (1971), but with lower

concentrations of purified synthetase and tRNA fractions. Unless otherwise stated the incubation mixture (0.1 ml) contained: ATP (64 nmol), MgCl₂ (500 nmol) and KCl (4 μ mol) in addition to radioactive amino acid, bean leaf synthetase and RNA fractions. The reaction mixture was incubated at 30°C for 30 min at pH 7.6.

Radioactivity measurement. The amount of isotopically labelled material adsorbed on the filter-paper discs used for the aminoacylation assay was measured by placing each disc in 5 ml of a scintillation cocktail containing 10 g of 2,5-diphenyloxazole, 0.3 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 150 g of naphthalene/l of 1,4-dioxan. The values reported were calculated from a liquid-scintillation counting efficiency of 17% for ³H and 74% (or 43% for a narrow window) for ¹⁴C on Whatman 3 MM paper discs (2.3 cm diameter).

pH values. All pH values stated were measured at the temperature of interest (4°C for dialysis buffers, 30°C for incubation mixtures). Values for very small volumes such as those used in aminoacylation reactions were obtained by spotting 5 μ l of solution on narrow-range pH papers (Macherey, Nagel und Co., Düren, Germany). The colour development of these papers was calibrated against values obtained for buffer solutions measured on a Corning model 12 pH-meter.

Controls. Valid results depended on the absence of microbial contamination from the incubation system, which was checked as a routine by culturing portions (0.01 ml) of the reaction mixture on nutrient agar slopes at 30°C for 48 h. Values reported in this paper are for incubations showing no microbial contamination by this test. A second control was the omission of ATP from the incubation system. Values similar to zero-time blanks were obtained for reaction mixtures without ATP when microbial contamination was absent. All results are the mean of at least two replicates.

RESULTS

Effect of precipitation of tRNA on amino acid acceptance. Phenol was separated completely from the RNA after filtration of the phenol-saturated aqueous layer through a Sephadex G-50 column (Fig. 1). Fig. 2 shows that RNA eluted from the column and concentrated with Aquacide II (method 2) could be charged with amino acids to a markedly greater extent than RNA prepared by ethanolic precipitation (method 1).

Deacylation of tRNA on Sephadex columns. The greatly increased capacity of tRNA prepared by Sephadex treatment for accepting amino acids indicated that this procedure was very efficient in deacylating the tRNA as well as in removing phenol from the solution. We were, therefore, interested in evaluating the extent to which endogenously charged amino acids were removed from tRNA on the column.

RNA prepared by method 2 was aminoacylated with a ¹⁴C-labelled amino acid mixture *in vitro* by using reaction mixtures scaled up to a final volume of 1.0 ml. After 30 min incubation the mixtures

Scheme 1. Preparation of aminoacyl-tRNA synthetase fractions.

Fig. 1. Removal of phenol from aqueous extracts of RNA by Sephadex G-50 column chromatography. A phenol-saturated aqueous extract of RNA (15 ml) was applied to a column (1.5 cm $\times$ 70 cm) and eluted with 20 mM-tris-HCl buffer (pH 7.5) containing 375 mM-NaCl and 8 mM-MgCl₂; 100-drop fractions were collected.

were dialysed at 4°C against 0.1 M-sodium phosphate buffer, pH 6.5, containing 12.5 mM-MgCl₂ to remove free amino acids and excess of ATP, which would prevent monitoring the column effluent in

the u.v. The radioactivity in the dialysis buffer was measured, then the buffer was changed until the counting rate decreased to 0.2% of that from the first change (from 99 866 c.p.m. to 219 c.p.m./0.1 ml of 100 ml total diffusate), indicating that most of the free amino acids had been removed from the dialysis bag. The radioactivity of the non-diffusible aminoacyl-tRNA (3.0 ml) was 1770 c.p.m./0.1 ml. Portions (1 ml) of the charged RNA were applied to Sephadex G-50 columns (8 mm $\times$ 200 mm) and eluted at 25°C with an acidic buffer [0.1 M-sodium phosphate (pH 6.5)-8 mM-MgCl₂-375 mM-NaCl] or with an alkaline buffer [0.02 M-tris-HCl (pH 7.5)-8 mM-MgCl₂-375 mM-NaCl]. Under alkaline conditions almost 91% of the radioactivity was removed from the tRNA, compared with the tRNA eluted with acid buffer, showing that less than 10% of the endogenously charged amino acids remain after this treatment (Fig. 3). The results from this experiment also indicated that the trailing edge of the RNA peak eluted from the Sephadex G-50 column contained a high proportion of the tRNA, since the u.v.-absorption peak preceded the peak of radioactivity resulting from amino [¹⁴C]acyl-tRNA (K. L. Tao & T. C. Hall, unpublished work).

Fig. 2. Effect of precipitation of tRNA on amino acid acceptance. The conditions were as described in the Experimental section, with the pH5 synthetase enzyme (50 μ g), 14 C-labelled amino acid mixture and tRNA prepared by various methods: $\square$, tRNA (30 μ g) prepared by method 1; Δ , tRNA (8 μ g) prepared by method 2; $\circ$, incubation mixture minus tRNA; $\bullet$, boiled enzyme.

DEAE-Sephadex A-50 chromatography. The deacylated RNA eluted from the Sephadex G-50 column was further purified by DEAE-Sephadex A-50 chromatography. Three major peaks were obtained by using a gradient of 375 mM–1 M-sodium chloride (Fig. 4), peak 1 having the highest amino acid acceptor capability (Table 1).

Effect of enzyme purification on the aminoacylation reaction. Adsorption of synthetase enzymes on calcium phosphate gel is an effective technique for their purification (Attwood & Cocking, 1965; Stern & Mehler, 1965). It was evident (Table 2) that not all synthetases were eluted in any one fraction from the gel (Scheme 1). In particular, alanyl- and phenylalanyl- tRNA synthetases were absent from fraction A but present in fraction B.

DISCUSSION

Preparation of tRNA. The procedure of Holley *et al.* (1961), with phenolic extraction (Kirby, 1956) of RNA for the preparation of tRNA, has proved to be an effective technique for many organisms, including plants (Vanderhoef *et al.*

1970). Although the RNA in the aqueous extract is usually isolated by addition of ethanol, the resulting precipitate is frequently difficult to dissolve (Johnson & Young, 1969). This is particularly true for extractions with sodium dodecyl sulphate as a ribonuclease inhibitor (Ingle, Key & Holm, 1965) and potassium acetate as an additional reagent during ethanolic precipitation. Since K^+ ions precipitate sodium dodecyl sulphate (Maizel, 1969) the complex of sodium dodecyl sulphate, RNA and K^+ is unlikely to be readily soluble. The results shown in Fig. 2 show a much higher amino acid-acceptor activity for RNA prepared without recourse to precipitation.

The use of Sephadex gel in the preparation of RNA fractions was noted previously (Gross, Skoczylas & Turski, 1965; McCoy & Carter, 1968); however, its application to removal of phenol in the preparation of tRNA appears to have been neglected. The high affinity of Sephadex for phenol (Determann & Walter, 1968) no doubt accounts for the effective separation of large amounts of RNA from phenol that can readily be accomplished by this procedure.

The use of Sephadex G-50 in the separation of tRNA is also of great value in that it can strip endogenous amino acids from the tRNA at pH 7.5 (Fig. 3). However, very little deacylation occurs when aminoacyl-tRNA was eluted at pH 6.5. The lack of deacylation under acidic conditions is in agreement with the finding of Vanderhoef *et al.* (1970) with soya-bean preparations.

Preparation of synthetase enzymes. The lability of aminoacyl-tRNA synthetase enzymes from plant sources demands that particular care be exercised to prevent degradation during extraction. The disruption of leaf cells permits rapid protein degradation by oxidative systems, especially polyphenol oxidases (Pirie, 1959; Pierpoint, 1969). Loomis & Battaile (1966) and Anderson (1968) documented the value of insoluble polyvinylpyrrolidone in removing phenolic compounds from plant extracts. Evidence for the removal of low-molecular-weight phenolic compounds by Dowex resins was presented by Lam & Shaw (1970), and Sephadex gels are also of value in removing relatively small molecules such as amino acids which result in dilution of supplied amino acids in the aminoacylation of tRNA (Attwood & Cocking, 1965). Residual amino acids also decrease the apparent activity of synthetase enzymes when assayed by pyrophosphate exchange (Anderson & Fowden, 1970). The powerful reducing agent dithiothreitol was introduced by Cleland (1964), and has been particularly valuable in the preparation of enzymes from plant tissues (Hall, McCown, Desborough, McLeester & Beck, 1969). The thiol protectant GSH has been shown to stabilize seryl-

Fig. 3. Deacylation of tRNA on Sephadex G-50 columns. RNA prepared by method 2 was charged with a ^{14}C -labelled amino acid mixture *in vitro* by using a reaction mixture scaled up to a final volume of 1.0 ml. After 30 min incubation the mixtures were dialysed at 4°C against 0.1 M-sodium phosphate buffer (pH 6.5) containing 12.5 mM- MgCl_2 for 2 days, the buffer being changed until the counting rate of the diffusate dropped to 0.2% of that of the first change. Portions (1 ml) of the dialysed charged tRNA were applied to Sephadex G-50 columns (8 mm $\times$ 200 mm) and eluted at 25°C with 0.1 M-sodium phosphate (pH 6.5)-8 mM- MgCl_2 -375 mM-NaCl buffer (O, E_{260} ; Δ , radioactivity) or with 0.02 M-tris-HCl (pH 7.5)-8 mM- MgCl_2 -375 mM-NaCl buffer ($\bullet$, E_{260} ; $\blacktriangle$, radioactivity). The eluates were collected in 10-drop fractions.

Fig. 4. Chromatography of RNA on DEAE-Sephadex A-50. The procedure followed was that given for RNA purification by method (4) in the Experimental section.

tRNA synthetase from yeast (Bluestein, Allende, Allende & Cantoni, 1968) and was also included in the extraction system. Flushing with N_2 is a convenient method for removing O_2 from the extraction

system. We have noted in other experiments that plant extracts can turn brown within 15 s when exposed to air, especially when insufficient reducing reagent is supplied. The excellent stability and high activity of the synthetase enzyme fractions obtained in the present study is attributed to the combination of the above ingredients, effectively buffered at pH 7.4 with Hepes (Good *et al.* 1966) which protect the extracted proteins until they are adsorbed on to the calcium phosphate gel.

The enzyme that was eluted from calcium phosphate gel at pH 7.6 (fraction A) gave a rate of 565 pmol of amino acid charged/min per mg of protein with an amino acid mixture and tRNA prepared by method 2 (Table 2). This rate was 20 times better than that catalysed by a pH 5-enzyme fraction (Fig. 2) and 665 times higher than that recorded by Hall & Cocking (1966) with a synthetase enzyme preparation from tomato leaves and ^{14}C -labelled protein hydrolysate.

It is notable that alanyl-tRNA and phenylalanyl-tRNA synthetases were present in the pH 6.5 eluate from the calcium phosphate gel, but absent from the pH 7.6 eluate. However, the synthetases

Table 1. *Extent of charging of tRNA purified by DEAE-Sephadex A-50 chromatography*

Synthetase enzyme fraction A (Scheme 1) (2.5 μ g), was added to the standard reaction system containing 14 C-labelled amino acid mixture and tRNA eluted from a DEAE-Sephadex A-50 column (Fig. 4).

tRNA fraction used	Amount of amino acid charged (pmol)	Amount of tRNA present (μ g)	Amount of amino acid charged (pmol/ μ g of tRNA)	Proportion of tRNA charged (%)
Peak 1	3.36	0.16	21.00	52.51
Peak 2	2.40	1.7	1.41	3.52
Peak 3	0.54	1.6	0.34	0.84

Table 2. *Aminoacyl-tRNA formation catalysed by different enzyme fractions*

Synthetase enzyme fractions A and B (Scheme 1) were tested for their ability to catalyse aminoacyl-tRNA formation in the assay system described in the Experimental section. Each assay contained 5 μ g of enzyme protein. When the 14 C-labelled amino acid mixture was supplied as a substrate 14 μ g of tRNA (prepared by method 2) was present, and 1.8 μ g of tRNA (prepared by method 3) was present in the assays with individual amino acids.

Amino acid substrate	Enzyme fraction	Amount of amino acid charged	
		(pmol/assay mixture)	(pmol/min per mg of protein)
Amino acid mixture	A	84.8	565
	B	40.2	267
Alanine	A	0	0
	B	1.4	9.2
Aspartic acid	A	1.3	8.8
	B	0	0
Lysine	A	1.6	10.7
	B	0	0
Proline	A	1.9	12.4
	B	0	0
Phenylalanine	A	0	0
	B	0.1	0.4

for aspartic acid, lysine and proline were present in pH 7.6 eluate and absent from the pH 6.5 eluate.

Importance of aseptic techniques. The importance of avoiding microbial contamination in cell-free extracts used for studies on aspects of protein synthesis has been stressed (Hall & Cocking, 1966; Wheeldon, 1966). As mentioned in the Experimental section, three controls were always run for the experiments reported in the present paper: zero time, a 'minus-ATP' incubation, and inoculation of an agar slope. Occasionally contamination was observed, and in all such cases the 'minus-ATP' incubation gave significantly higher values than the zero-time control. Calculation of the extent of charging in these instances suggests that more than one molecule of amino acid was attached per molecule of tRNA; i.e. there was greater than 100% charging, assuming one molecule of amino acid per molecule of tRNA is the maximum extent to which tRNA can be aminoacylated. Since the number of moles of tRNA present in the incubation was calculated by dividing the weight of RNA present

in the mixture by a molecular weight of 25000, one explanation for apparent charging of over 100% could be the presence of tRNA fragments of molecular weight less than 25000. However, it would appear advisable that any experiments giving such values should be checked for microbial contamination as well as considering the possibility of charging tRNA fragments.

Extent of charging of tRNA in vitro. The results presented in Fig. 3 support our previous contention (Hall & Tao, 1970) that endogenously bound amino acids can give rise to apparently low extents of aminoacylation of tRNA *in vitro* because they effectively dilute the specific radioactivity of the supplied radioactive amino acid. The lack of a requirement for exogenously supplied amino acids for protein synthesis noted for a cell-free system from tobacco (Sela, 1969) could also arise from the presence of endogenously charged amino acids in the extract. Deacylation of endogenously bound amino acids by Sephadex G-50 chromatography under alkaline conditions is a valuable step in

obtaining demonstrably active aminoacylation of tRNA *in vitro*.

Further purification of the RNA eluted from the Sephadex G-50 column by DEAE-Sephadex A-50 chromatography yielded three peaks (Fig. 4). In studies on tobacco mosaic virus-infected tobacco leaves Johnson & Young (1969) obtained three peaks from DEAE-cellulose chromatography, and found amino acid-acceptor activity present in peak 3. Electrophoresis of peak 3 of the tobacco leaf RNA was reported by the above authors to yield a faint slow-moving and a strong faster-moving band. Electrophoresis of bean leaf RNA of 2.5% (w/v) acrylamide gels also yielded more than one component for peak 3 (A. R. Rastogi & T. C. Hall, unpublished work), the three bands observed corresponding to approx. 5S, 20S and 22S. Single bands were obtained for RNA from peaks 1 and 2, that from peak 2 running a little slower than that from peak 1 (corresponding to about 4.5S and 4S respectively).

Although further characterization of the peaks obtained from DEAE-Sephadex chromatography is necessary, it is of importance that RNA from peak 1 (Fig. 4) could be charged with an amino acid mixture to 52% of the theoretical maximum *in vitro* (Table 1). Calculation of the results for aminoacylation of tRNA from peak 1 (Table 1) on an RNA basis gives a rate of 0.7 nmol of amino acid incorporated/min per mg of RNA. This rate is three orders of magnitude higher than obtained previously (see Table 6 of Hall & Tao, 1970) and is reasonably close to the rate observed *in vivo*. The preparation of tRNA capable of being charged to this extent will be of great value in studies concerned with the binding of tRNA to the ribosome and subsequently for obtaining meaningful incorporation of amino acids into protein *in vitro*.

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