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PURIFICATION OF TRANSFER RNA AND STUDIES ON AMINOACYL-tRNA SYNTHETASES FROM HIGHER PLANTS*

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Abstract—Transfer RNA was purified from pea root and soybean hypocotyl by selective dissolution in 3·0 M sodium acetate and subsequent DEAE-cellulose chromatography. The product was characterized by u.v. absorbance, gel electrophoresis, aminoacylation, and two tests for RNAse damage (melting and reannealing, and characterization of the 3′-CpCpA terminus). Aminoacyl-tRNA synthetases were extracted and purified using ammonium sulfate fractionation and calcium phosphate gel batch chromatography. RNAse, ATPase, pyrophosphatase and -CpCpA pyrophosphorylase activities were assayed in the synthetase preparation. Optimum pH, conc. $MgCl_2$ and conc. ATP were determined for the aminoacylation of six amino acids to their respective tRNAs. The reaction was further characterized by determining the kinetics of aminoacylation with increasing (but always limiting) tRNA conc., conc. amino acid and conc. synthetase preparation.

INTRODUCTION

TRANSFER RNA (tRNA) is intimately involved in the first reaction in the assembly of protein, being responsible for the proper translation of the sequence of bases in messenger RNA (mRNA) into the prescribed order of amino acids in protein.[1] This molecule, along with the tRNA synthetases, is central in theories of translational regulation of protein synthesis.[2–10] Several methods have been developed for the isolation of tRNA.[11–16] The most popular method is Holley's,[16] which utilized the phenol extraction of Kirby,[17] and subsequent fractionation on DEAE-cellulose, employed first for this purpose by Zamecnik.[12] The method

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gave a relatively pure and active product when extracted, for instance, from yeast[16] or *Neurospora.*[18] But with *Escherichia coli* the final sRNA product was 25% mRNA and rRNA.[19] With higher plants the Holley method was also inadequate, the final product being only 20% as pure as that from yeast,[20] based on $Å_{260}$/mg. A similar anomaly existed for Zubay's extraction procedure.[21] This process utilized, after a phenol extraction, the differential extraction of sRNA from the total RNA pellet with 1·0M NaCl.[22] The procedure, with *E. coli*, gave a relatively pure and active product. Again, however, the method did not give good results with plant or animal tissue, since 1·0 M NaCl extracted considerable high mol. wt. RNA (HMW RNA, including rRNA and mRNA) yielding a final product from 40 to 60% rRNA in the case of pea roots and soybean hypocotyl,[23] 50% in beet root[24] and 35% in rat liver.[25] In spite of these shortcomings, these methods have been used by plant physiologists[20, 26–28] for lack of better methods. The method described herein utilizes phenol extraction, differential solubility of the RNA in 3·0 M NaOAc[29] and DEAE-cellulose chromatography.[12] The product is as pure as any thus far characterized from mammals and bacteria.

Aminoacyl-tRNA synthetases catalyze the two-step reaction in which an amino acid is attached to tRNA. Synthetase assay methods have been discussed in detail;[1, 30–33] aminoacyl hydroxamate formation, amino acid-dependent ATP–PP exchange and formation of isotopic amino acid-tRNA have been most widely used. The first two methods lack strict specificity, and each gives a high endogenous background, while strict specificity and low background are characteristics of the measurement of attachment of amino acids to tRNA.[28, 31, 34] Synthetases have been partially purified from several plant tissues, and as early as 1958 Clark demonstrated amino acid activation by extracts from spinach leaves and pea epicotyls.[35] The most successful purifications have employed some combination of ammonium sulfate precipitation, calcium phosphate gel fractionation, and ion-exchange chromatography, and were first used for plant synthetases by Moustafa,[28, 36–39] and subsequently by Fowden[40–43] and Cocking.[44]

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This paper reports the purification and partial characterization of tRNA from plants and the partial purification of aminoacyl-tRNA synthetases from soybean hypocotyl and pea root. The purification was designed to yield a preparation containing activity of a broad spectrum of synthetases, but sufficiently free of extraneous enzymes such as RNAase to prevent complications in studies of the charging reaction. The aminoacylation reaction was also studied in some detail.

RESULTS AND DISCUSSION

Transfer RNA Purification

The phenol extraction of nucleic acids in the absence of a detergent, such as sodium lauryl sulfate, yielded a final product containing a relatively small percentage of the total DNA and D-RNA.[45] Thus, a partial purification of tRNA was effected in the initial homogenization. Low mol. wt. RNA, including tRNA and 5S rRNA (LMW RNA), has been separated from high mole. wt. RNA (HMW RNA) (primarily 25 and 18S rRNAs) by several techniques, e.g. methylated Albumin Kieselguhr column chromatography,[46] sucrose gradient centrifugation,[47] gel filtration,[48] and differential solubility of cetyltrimethylammonium RNA salts.[49] On a preparative scale differential solubility in salt solutions[50,51] presented the most attractive approach. HMW RNA was effectively separated from LMW RNA in conc. NaOAc of 2·5 M or higher (Fig. 1). When total RNA from pea roots was suspended in 0·25

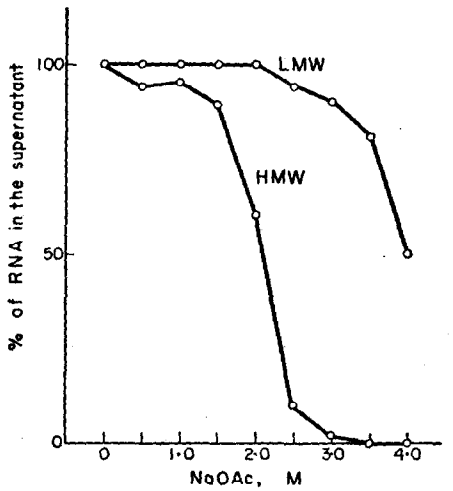

FIG. 1. THE FRACTIONATION OF PEA ROOT RNA IN SODIUM ACETATE SOLUTIONS.

Total RNA was thoroughly suspended in the various NaOAc solutions, and stored at 4° for 6–12 hr. After separation by centrifugation the size distribution of the soluble and insoluble RNA was determined by measuring the total amount of RNA in the supernatant and in the precipitate, along with gel electrophoresis of both fractions. As an example, with 3·0 M NaOAc there were 15·63 $Å_{260}$ in the supernatant (14·00 LMW, 1·63 HMW), while in the precipitate there were 82·15 $Å_{260}$ (1·55 LMW, 80·60 HMW).

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tissue vol. 3·0 M NaOAc, 90% of the LMW RNA was dissolved, while only 2% of the HMW RNA was dissolved. On an absolute basis, this meant that for every 20 $Å_{260}$ of LMW RNA in the supernatant, there were 1·5 $Å_{260}$ of HMW RNA. All of the HMW RNA remained precipitated when the pellet was suspended in 3·5 M NaOAc (Fig. 1); however, about 20% of the LMW RNA was not dissolved at this conc. NaOAc (conc. 3 M) was routinely used for the primary separation of LMW and HMW RNAs. It was found later that a ratio of 1 ml of 3 M NaOAc:2 mg total RNA was optimum for this fractionation. If LMW RNA was precipitated from the 3·0 M NaOAc supernatant with 2·5 vol. ethanol at 4° or colder, NaOAc

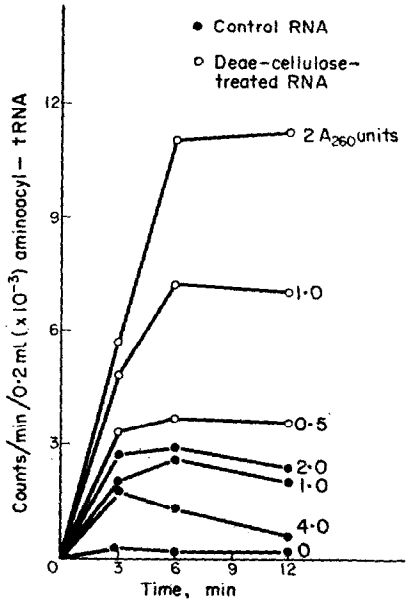

FIG. 2. THE PURIFICATION OF LMW RNA WITH DEAE-CELLULOSE—ITS EFFECT ON AMINOACYLATION OF tRNA.

The precipitate obtained from the supernatant 3·0 M NaOAc was dissolved in buffer I and adsorbed on a 1 × 3 cm gravity-settled DEAE-cellulose column, previously equilibrated in buffer I. A 5-ml wash with buffer I was followed by elution with 7 ml buffer II. The LMW RNA was precipitated with 17·5 ml ethanol. The precipitate was washed twice with ethanol, then dissolved in 0·001 M NaOAc, pH, 4·5 containing 0·001 M $MgCl_2$. Aminoacylation was accomplished by the method described in the text.

crystallized out. This was avoided by diluting the 3·0 M NaOAc with 1 vol. H_2O prior to precipitation.

LMW RNA at this stage of purification was relatively free of other species of nucleic acids, but contained some protein, a great deal of carbohydrate material, and possibly other contaminants. It was discovered early in this study that materials in the LMW RNA preparation inhibited the charging reaction (aminoacylation of tRNA) (Fig. 2). At limiting concentrations of LMW RNA (data not included) the increase in the initial rate of aminoacylation was linear with the increase in RNA concentration up to 0·8 $Å_{260}$/ml; above 0·8 $Å_{260}$ of RNA the initial rate of the reaction decreased with increasing RNA concentration, approaching zero at about 8 $Å_{260}$/ml. This inhibition prompted the use of DEAE-cellulose for further purification of the LMW RNA preparation. The data of Fig. 2 illustrate the effects of this

purification on the acylation of tRNA. The level of charging of tRNA was now proportional to the amount of tRNA used. In addition, this procedure eliminated the remaining HMW RNA and traces of DNA from the tRNA preparation (Fig. 3). A u.v. scan of the final LMW RNA product [see Fig. 3(D)] and relevant wavelength ratios are shown in Fig. 4. The final preparations gave 16–18 $Å_{260}$ units per mg. All results indicated that the LMW RNA was of high purity.

Several parameters were checked in attempts to ascertain if the tRNA molecules were damaged during isolation (e.g. nicked by RNAase). Pea root transfer RNA was "melted" at

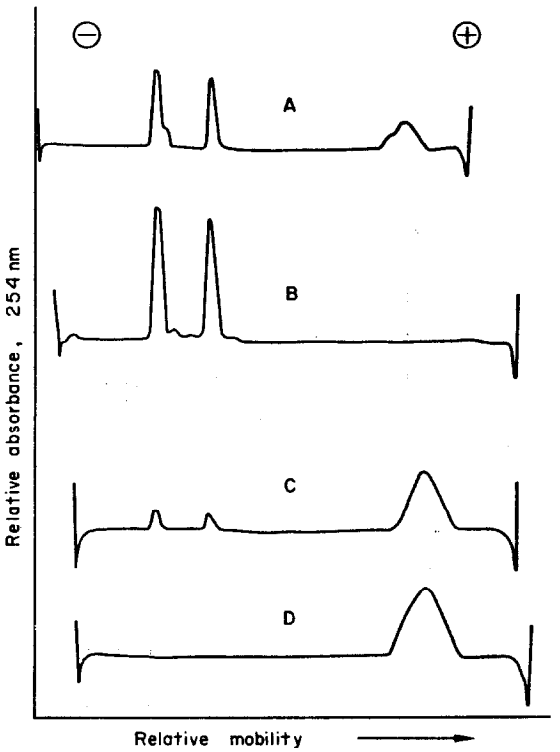

FIG. 3. THE PURIFICATION OF LMW RNA.

RNA was analyzed by gel electrophoresis at four stages in the purification: (A) Total RNA, (B) that RNA which did not dissolve in 3·0 M NaOAc, (C) that RNA which dissolved in 3·0 M NaOAc, and (D) fraction C after purification on DEAE-cellulose.

85° and then quick-cooled, the logic being that if there were RNAase "nicks", melting would separate the fragments, and quick-cooling would not allow proper reassociation to form an active tRNA molecule. If there was no RNAase damage, this process would result in re-association into an active tRNA molecule. The latter was found to be the case, e.g. the melting and reannealing had no effect on the charging capacity of tRNA (see Fig. 5 for leucyl tRNA). Similarly, soybean tRNA was heated and cooled to room temp. with no significant change in the level of tRNA charged for six different amino acids. Further evidence for the lack of RNAse damage of the tRNA during the extraction procedure was found in the characterization of the 3′-CpCpA terminus. Approximately 90 per cent of the tRNA molecules from pea root (84 per cent for soybean hypocotyl tRNA) had adenosine as the terminal

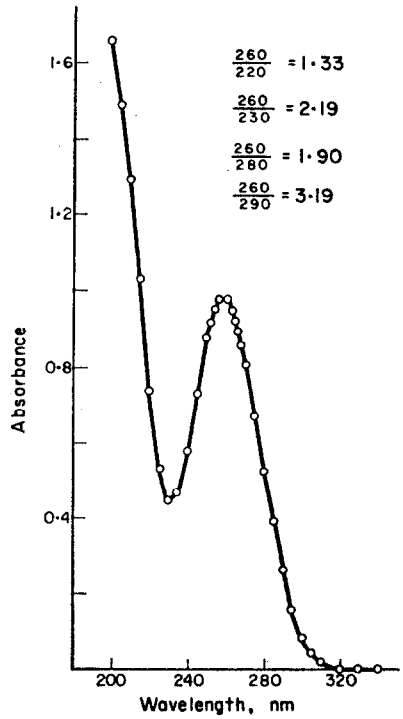

FIG. 4. U.V. SPECTRUM OF PEA ROOT LMW RNA.
LMW RNA was purified as described in the text. A Zeiss PMQ II spectrophotometer was used to determine the u.v. absorbancy of a 0·005% solution (w/v).]

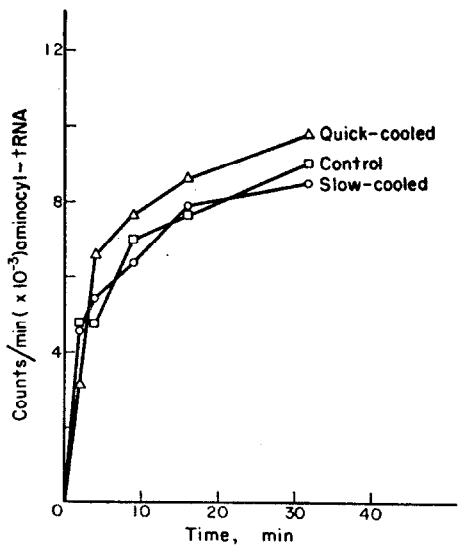

FIG. 5. THE EFFECT OF MELTING ON THE CHARGING CAPACITY OF tRNA.
Two $Å_{260}$ LMW RNA were dissolved in 10^{-3} M $MgCl_2$, containing 10^{-3} M NaOAc, pH 6·5, and slowly heated to 85° in a water bath, and held there for 15 min. The RNA was then either quick-cooled (transferred to an ice bath) or slow-cooled (transferred to the bench top). The two tRNA samples, along with a control sample, were then charged with 0·5 μc ^{14}C-leucine as described in the text.

nucleoside, and, presumably then, an intact -CpCpA terminus (Fig. 6). The high amount of uridine (about 10% of the total nucleosides following alkaline hydrolysis of the RNA) was attributed to 5S ribosomal RNA,[52,53] which makes up about 10% of the LMW RNA preparation of pea and soybean, as determined by gel electrophoresis on 5% polyacrylamide gels.

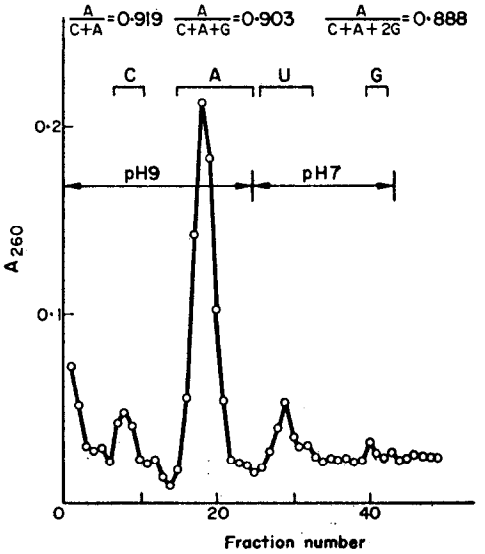

FIG. 6. THE FRACTIONATION OF NUCLEOSIDES FROM BASE-HYDROLYZED LMW RNA.

Forty $Å_{260}$ pea root LMW RNA were hydrolyzed in 0·3 N KOH at 37° for 18 hr. The hydrolysate was chilled and adjusted to pH 9 with 2·4 N perchloric acid. The insoluble material was centrifuged out and the nucleosides were separated as described in the text. One-ml fractions were collected.

TABLE 1. THE EFFECT OF DEACYLATION OF tRNA ON THE AMINO ACID ACCEPTANCE LEVELS BY tRNA EXTRACTED AND PURIFIED AT pH 7·5 AND AT pH 5·5

	Amino acid					
	Ser	Leu	Lys	Phe	Tyr	Met
pH 7·5						
Before deacylation	2553	3208	1252	1762	2650	2481
After deacylation	2628	3174	1195	1690	2526	2357
pH 5·5						
Before deacylation	2270	2261	950	800	1665	1798
After deacylation	2905	3266	1629	1426	1848	2645
% Increase	22	31	42	44	10	32

Transfer RNA was deacylated and charged to saturation as described in the text. Values are counts/min ^{14}C-amino acid charged/0·02 mg tRNA. The two types of experiments were performed on separate days with different enzyme preparations.

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When soybean RNA was extracted at pH 7·5 from tissue which had been cut into cold H_2O, the final tRNA product was completely "stripped" (i.e. no aminoacyl tRNA remained); however, when the tissue was cut and immediately homogenized, and the entire extraction procedure kept at pH 5·0–5·5, approximately 30 per cent of the tRNA molecules were aminoacylated (Table 1). Because a "stripped" product was preferred in these studies, the extraction was usually performed at pH 7·5.

The Extraction and Estimation of Aminoacyl-tRNA Synthetases

The method of Moustafa[36–39] employed protein fractionation by ammonium sulfate precipitation and calcium phosphate gel fractionation; these methods were investigated for use with pea root and soybean hypocotyl. Protein which precipitated between 0·4–0·7 $(NH_4)_2SO_4$ saturation contained from 70% (leucine) to 100% (serine) of the total synthetase activity in the pea root homogenate and from 90% (phenylalanine) to 100% (leucine) of the total synthetase activity in the soybean hypocotyl homogenate (Table 2).

TABLE 2. FRACTIONATION OF THE 30,000 *g* SUPERNATANT WITH AMMONIUM SULFATE

$(NH_4)_2SO_4$ Saturation (%)	Synthetase activity					Lowry protein	
	Ser	Leu	Lys	Phe	Tyr	mg/ml	% Total
0–40	0	0	1	10	4	5·5	49
40–70	95	100	98	90	93	5·5	49
70–95	5	0	1	0	3	0·2	2
0–45	0	10	15	11	5	7·5	63
45–65	77	83	84	86	74	3·4	29
65–95	23	7	1	3	21	1·1	9

The homogenate was centrifuged at 30,000 *g* for 30 min and the supernatant fractionated by the addition of crystalline ammonium sulfate. Protein precipitating over each saturation range was dissolved in 10 ml of pH 7·5 0·01 M HEPES buffer containing 0·0005 M dithiothreitol. A 0·1 ml aliquot was assayed in a 1 ml reaction mixture as described in the text. The 1·0 ml reaction volume contained 100 μmole/HEPES, pH 7·5, 5μmole/$MgCl_2$, 1 μmole ATP, 3 mg yeast tRNA, 0·5 μc ^{14}C-amino acid (50 mc/mmole). Amino acid and tRNA were in excess. The initial rates were determined and summed, and the values reported are percentages of that sum in each fraction for each amino acid.

The purification of the enzyme preparation with calcium phosphate gel indicated that a large amount of gel suspension, 0·8 vol., was necessary to adsorb all of the pea root serine synthetase activity while all of the pea root tyrosine synthetase activity was adsorbed from the same protein solution with only 0·1 vol. gel suspension (Fig. 7). In comparison, soybean hypocotyl synthetases were almost completely adsorbed by only 0·5 vol. gel (Table 3). A summary of the purification of soybean synthetases is shown in Table 4.

The data reported in Table 5 show the requirement for a reducing agent in the extraction medium; however, it is apparent that not all the synthetases lose activity in the absence of reducing agent. It was shown that active synthetases could be extracted from pea roots which had been frozen and stored on dry ice for up to 2 weeks. However, both pea root and soybean synthetases decreased in activity when the purified enzyme preparation was stored at –20°. Dialysis of the preparations likewise resulted in loss of synthetase activities.

Several enzymes which might indirectly affect the charging of tRNA, in addition to the synthetases, were assayed in the final synthetase preparation. Data from these experiments

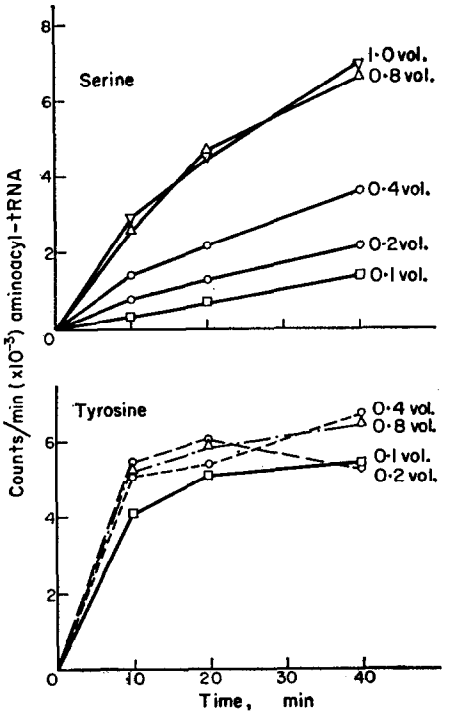

FIG. 7. THE SYNTHETASE ACTIVITY OF THE PROTEIN ADSORBED ON VARIOUS AMOUNTS OF CALCIUM PHOSPHATE GEL.

The protein precipitated from 0·4 to 0·7 ammonium sulfate saturation was dissolved in 0·01 M HEPES, pH 7·5, containing 0·0005 M dithiothreitol. Increasing voulmes of calcium phosphate gel suspension (20 mg/ml) were added dropwise to the stirring protein solution for adsorption of protein. The enzyme was subsequently eluted from the gel and re-dissolved in the HEPES–dithiothreitol solution. Assay for synthetase was as described in the text.

TABLE 3. THE PURIFICATION OF SOYBEAN HYPOCOTYL SYNTHETASES BY CALCIUM PHOSPHATE GEL FRACTIONATION

	Synthetase activity					Lowry protein	
	Ser	Leu	Lys	Phe	Tyr	mg/ml	% Total
Activity adsorbed to 0·4 vol.* gel	89	80	97	82	78	2·12	65
Activity adsorbed to 0·5 vol. gel	98	100	100	100	95	2·40	73

* 1 vol. = 1 ml gel suspension 20 mg/ml/g fr. wt.

Calcium phosphate gel was added to the 40–70% ammonium sulfate fraction; that protein which adsorbed was subsequently eluted and assayed for synthetase activity. Initial rates were determined and the values presented are percentages of total synthetase activity (as determined by measuring the activity in the protein solution prior to calcium phosphate gel treatment) which adsorbed to the gel.

are summarized in Table 4. RNAse activity was not detected under conditions of the synthetase assay when a technique which measured solubilized radioactive RNA (pieces of RNA released by nuclease activity which are small enough to be soluble in cold 10% TCA) was used. However, endonuclease activity was detected when the LMW RNA which had

been incubated with large amounts of enzyme preparation was subsequently fractionated on 5% polyacrylamide gels. After 60 min most of the 5S rRNA and a small fraction of the 4S RNA had been nicked yielding pieces which migrated in the 3S region of the gel (about 40 nucleotides long).

TABLE 4. SUMMARY OF THE PURIFICATION OF AMINOACYL-tRNA SYNTHETASES FROM SOYBEAN HYPOCOTYL

		Purification step		
		Crude	$(NH_4)_2SO_4$	$Ca_3(PO_4)_2$
		μmoles AA charged/min/mg protein		
Synthetase for:	Ser	31·0	372	1075
	Leu	19·0	97	222
	Lys	52·5	299	735
	Phe	16·5	166	531
	Tyr	140·0	256	562
	Met	40·0	64	238
		Parameter Measured		
Protein:	Total mg in preparation	131	41·6	9·7
	μg/charging assay	408	416	194
RNA:	Total mg in preparation	9·92	0·11	0·05
	μg/charging assay	31	11	10
RNAase:	μg RNA hydrolyzed/min/mg protein	—	5·8	0
ATPase:	mg Pi released/hr/mg protein			7·60
Pyrophosphatase:	mg Pi released/hr/mg protein			4·42
pCpCpA Pyrophosphorylase:	μmoles ATP incorporated/min/mg protein			
	−CTP			0·77
	+CTP			1.50

Protein at various purification stages was assayed for various enzymes. Synthetase assays were performed under conditions described in the text. RNAase, ATPase and pyrophosphatase assays were performed under charging conditions with ^{14}C- or ^{3}H-RNA, ATP or pyrophosphate as substrates, respectively. The pCpCpA pyrophosphorylase assays were performed under optimal *E. coli* pyrophosphorylase conditions.[54] Incorporation of ^{14}C-ATP into yeast tRNA with and without added CTP was measured.

TABLE 5. THE INFLUENCE OF DITHIOTHREITOL ON ENZYME EXTRACTION FROM SOYBEAN HYPOCOTYL

Extraction	Synthetase activity					
	Ser	Leu	Lys	Phe	Tyr	Met
	counts/min charged/min/40 μg protein					
With dithiothreitol	2668	362	1140	418	844	289
Without dithiothreitol	2631	153	1040	166	684	60

Protein was assayed for synthetase activity following ammonium sulfate and phosphate gel fractionation and final dissolution in 0·01 M HEPES, pH 7·5. One preparation contained 0·0005 M dithiothreitol throughout, the other did not.

The experiments to evaluate -CpCpA pyrophosphorylase activity were designed to measure the incorporation of ^{14}C-ATP into yeast tRNA. By the same methods that the -CpCpA terminus was characterized for pea and soybean tRNA (Fig. 6), it was determined that only 25 per cent of commercial yeast tRNA molecules had an intact -CpCpA terminus;

[54] J. HURWITZ and J. FURTH, *Proc. Nucleic Acid Res.* (edited by G. CANTONI and D. DAVIES), p. 347, Harper & Roe (1966).

thus, such a preparation of tRNA was a good substrate for this enzyme. Under conditions optimum for synthetases, no activity was observed. Under optimum conditions for *E. coli* pyrophosphorylase,[54] however, a low level of activity was observed. ^{14}C-ATP was incorporated, and this incorporation was enhanced by the presence of CTP (Table 4).

ATPase activity was also assayed and found to be present in the synthetase preparation (Table 4). However, the level was so low that even if the initial rate were to continue throughout the reaction time, 40 min would have been required for all ATP to be hydrolyzed in the usual 1 ml reaction mixture. This level of activity apparently did not affect the charging of tRNA since charging level was proportional to the amount of tRNA present (Fig. 9).

There was sufficient pyrophosphatase present in the enzyme preparation (Table 4) to hydrolyze pyrophosphate as fast as it was formed in the aminoacylation reaction. It was assumed that this assisted in driving the reaction to completion and eliminated possible inhibition by pyrophosphate.[55]

Characterization of the Aminoacylation Reaction

When 0·5 μmole of each of five ^{14}C-amino acids (0·1 μc each of leucine, serine, lysine, phenylalanine and tyrosine) was present in a 1 ml reaction volume, the optimum conditions

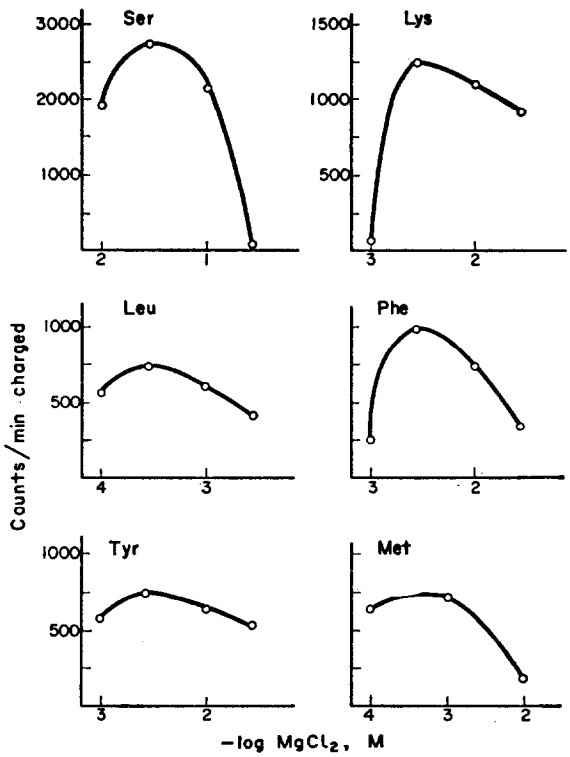

FIG. 8. THE OPTIMUM $MgCl_2$ CONCENTRATION FOR AMINOACYL-tRNA SYNTHETASES FROM SOYBEAN HYPOCOTYL.

The reaction mixture contained 0·01 mM ^{14}C-amino acid, 100 mM HEPES, pH 8·0, 1 mM ATP, excess tRNA and varying concentrations of $MgCl_2$. The initial reaction rate was determined at the various $MgCl_2$ concentrations for each of six amino acids.

[55] A. MEHLER, *Synthesis Structure and Function of Transfer RNA*, Miles Laboratory Symposium, New York (1968).

for aminoacylation of the soybean hypocotyl tRNAs were pH 8·0 (0·01 M HEPES), 1 mM ATP, and 5 mM $MgCl_2$. It was known, however, that these would not necessarily be the optimum conditions for the charging of individual amino acids since optimum Mg^{2+}:ATP ratios have been found to range from 1:1 to 1:50 for different synthetases from the same organism.[1] Accordingly the different parameters were measured for the individual synthetases. First, at 1 mM ATP and 5 mM $MgCl_2$ the optimum pH for five out of six soybean synthetases was pH 8·0 (serine, leucine, tyrosine, lysine, phenylalanine; methionine = pH 8·5).

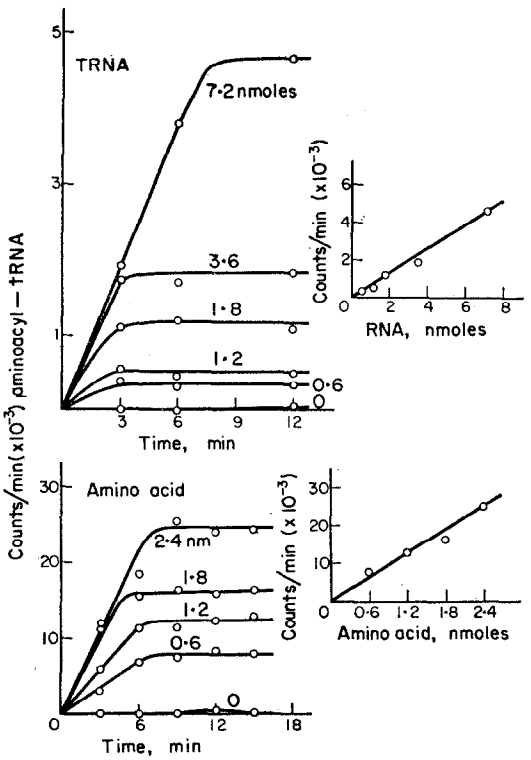

FIG. 9. THE EFFECT OF LMW RNA AND AMINO ACID CONCENTRATION ON TOTAL AMOUNT OF tRNA ACYLATED.

Both 1-ml reaction mixtures contained 100 μmole HEPES, pH 7·5, 1 μmole ATP, 5 μmoles $MgCl_2$, and approximately 0·3 mg protein. Where tRNA was limiting (upper graphs) 10 nmole ^{14}C-tyrosine were present. Where ^{14}C-tyrosine was limiting (lower graphs) 50 nmole LMW RNA were present. The graphs on the left are time course assays; the graphs on the right are plots of plateau values against substrate amounts.

Second, at pH 8·0 the optimum ATP conc. was 1 mM for all synthetases assayed. Finally, at pH 8·0 and 1 mM ATP, the optimum $MgCl_2$ concentrations were measured for six synthetases (Fig. 8). The initial rates were recorded, and optimum $MgCl_2$ conc. up to a 100-fold difference for different synthetases were measured: 50 mM for serine; 5 mM for lysine, phenylalanine, tyrosine, and methionine; and 0·5 mM for leucine.

The amount of tRNA charged, when present in limiting concentrations and in the presence of 0·1 μmole ^{14}C-amino acid, was linear with increase in the amount of tRNA present (see Fig. 9 for tyrosine data). This was also true when amino acid concentration was the

limiting component in the reaction and increasing amounts were added (Fig. 9). When enzyme was present in limiting amounts the increase in the initial rate of aminoacylation was linear with increase in enzyme concentration.

EXPERIMENTAL

Plant Tissue

Soybean seeds (*Glycine max*, Hawkeye 63) were germinated in moist vermiculite in the dark at 30° and 100% humidity. After 65 hr the apical 3 cm of the hypocotyls were excised for enzyme or tRNA extraction. Pea seeds (*Pisum sativum*, Alaska) were germinated between moist layers of absorbent paper at about 25°. After 96 hr the roots were excised for enzyme or tRNA extraction.

Transfer RNA Purification and Characterization

Total RNA was extracted according to Kirby;[29] tissue was homogenized in 1 tissue vol. (1 g = 1 ml) 0·01 M Tris-Cl buffer, pH 7·5, and 2 tissue vol. phenol (saturated with 0·01 M Tris-Cl buffer, pH 7·5) containing 10% cresol (v/v) and 0·1% hydroxyquinoline (w/v). After centrifugation of the homogenate at 17,000 *g* for 15 min, the aqueous layer was drawn off and repeatedly washed with equal vol. of Tris-saturated phenol (usually 4–5 times) until no protein appeared at the interface after centrifugation. The RNA was precipitated from the aqueous solution (made 0·15 M NaOAc, pH 6·0) by addition of 2·5 vol. of ethanol at −20°. The precipitate was pelleted by centrifugation at 5000 *g* for 5 min, and washed twice with cold absolute ethanol. After much experimentation (see Results and Discussion) the following procedure was used for fractionation of the RNA. The total RNA pellet was thoroughly suspended in ice-cold 3·0 M NaOAc, pH 6·0, followed by storage at 4° for at least 12 hr. The suspension was then centrifuged at 5000 *g* for 5 min. The pellet, containing the rRNA (Fig. 3), was discarded; the supernatant was diluted with 1 vol. H_2O and the RNA precipitated at 4° with 2·5 vol. absolute ethanol. This RNA was dissolved in buffer I (0·01 M NaOAc, pH 4·5, containing 0·3 M NaCl, 0·01 M $MgCl_2$, and 0·0005 M dithiothreitol) for further purification on DEAE-cellulose.[12] DEAE-cellulose was equilibrated in buffer I, and a 1 × 3 cm gravity-settled column was prepared. RNA, dissolved in buffer I, was adsorbed onto the column. The column was then washed with 6 ml buffer I to remove non-adsorbed materials, including polysaccharides, protein, and small oligonucleotides. The LMW RNA (4S tRNA and 5S rRNA) was then eluted with 7 ml buffer II (buffer I with 1·0 M NaCl). The RNA was precipitated from this solution with 2·5 vol. of absolute ethanol, collected by centrifugation, dissolved in 0·001 M NaOAc, pH 4·5, containing 0·001 M $MgCl_2$, and stored at −20° for future experiments. The DEAE-cellulose column and subsequent washing and elution steps were appropriately scaled up for amounts of RNA too large for the 1 × 3 cm column.

Gel electrophoresis, used to determine the optimum conc. NaOAc and vol. for fractionation of the RNAs (Fig. 1) and to monitor the entire fractionation procedure (Fig. 3), was performed according to Loening.[56]

End-group determination (ascertaining the identity of the 3′ terminal base) was performed according to the method of Jeffers and Gilham.[57] 2 mg LMW RNA were hydrolyzed in 1 ml 0·3 N KOH for 18 hr at 37°. The hydrolysate was chilled to 4° and adjusted to pH 9 with 2·4 N perchloric acid. The insoluble potassium perchlorate was removed by centrifugation, and the supernatant solution was added to a 0·45 × 30 cm BioRad AG1-X2 column (Cl^- form, 200–400 mesh), previously equilibrated in 0·05 M Tris-Cl, pH 9·0, containing 0·02 M KCl and 1% *n*-butanol (v/v). Cytosine and adenosine were eluted with separation with the pH 9·0 buffer. Uridine and guanosine were then eluted with separation with the same solution adjusted to pH 7·0. (Fig. 6). For comparison of nucleoside peaks total absorbance/peak was converted to a molar quantity.

Transfer RNA was deacylated by incubating for 30 min at 37° in 0·1 M Na_2CO_3, pH 10·0.[58]

Transfer RNA was "melted" by heating 2-ml samples (4 nmole RNA in 0·001 M NaOAc, pH 4·5, containing 0·001 M $MgCl_2$) slowly from 25° to 85°, then holding at 85° for 10 min.[59] Reannealing was accomplished by slowly cooling the solution back to 25°, or by quickly cooling to 0° by placing the sample in an ice bath.

Synthetase Purification and Characterization

Synthetases were extracted from tissue (apical 3 cm of soybean hypocotyls, entire roots of peas) according to a modified method of Moustafa. Tissue was minced with a razor blade in 1 tissue vol. 0·4 M sucrose containing 0·01 M $MgCl_2$, 0·0005 M dithiothreitol, and 0·01 M HEPES buffer, pH 7·5. The slurry was then homogenized in a Polytron homogenizer at low speed for 20–30 sec. The homogenate was centrifuged for

[56] U. Loening, *Biochem. J.* **102**, 251 (1967).
[57] J. Jeffers and P. T. Gilham, personal communication.
[58] P. Zamecnik, M. Stephenson and J. Scott, *Proc. Natl. Acad. Sci. U.S.* **46**, 811 (1960).
[59] J. Fresco, A. Adams, R. Ascione, D. Henley and T. Lindahl, *Cold Spring Harbor Symp. Quant. Biol.* **31**, 527 (1966).

30 min at 30,000 *g* and the supernatant passed through miracloth. Partial fractionation of proteins was accomplished by $(NH_4)_2SO_4$ precipitation by slowly adding crystalline $(NH_4)_2SO_4$ to the supernatant solution with continuous stirring. That protein which precipitated between 0·4–0·7 $(NH_4)_2SO_4$ saturation was collected by centrifugation (12,000 *g* for 10 min); the pellet was gently rinsed with distilled H_2O, then dissolved in 0·5 tissue vol., pH 7·5, 0·01 M HEPES buffer containing 0·0005 M dithiothreitol. Calcium phosphate gel (20 mg/ml), prepared according to Kunitz,[60] was then added dropwise to the stirring protein solution until the concentration of gel in the protein solution was 10 mg/ml for pea root (5 mg/ml for soybean hypocotyl). The gel and adsorbed protein were pelleted by centrifugation at 500 *g* for 5 min. The supernatant was discarded and the protein was eluted from the phosphate gel by thorough suspension in 0·01 M KH_2PO_4 (adjusted to pH 7·5 with KOH) containing $(NH_4)_2SO_4$ (15%, w/v, for the pea tissue, and 20%, w/v, for the soybean tissue). The gel was then pelleted by centrifugation and discarded. The protein was precipitated from the supernatant by slowly increasing $(NH_4)_2SO_4$ conc. to 0·8 saturation with crystalline $(NH_4)_2SO_4$. The precipitate was dissolved in 0·25 tissue vol. 0·01 M HEPES, pH 7·5, containing 0·0005 M dithiothreitol. All steps in the extraction procedure were carried out at 0–4°.

Protein determinations were by the Lowry method,[61] using bovine serum albumin as a standard, or by the Kalckar formula.[62] The amount of RNA in the synthetase preparation was measured according to previously published methods.[63] RNA was precipitated with perchloric acid, the pellet washed, and the RNA hydrolyzed in 0·3 N KOH at 37° for 18 hr. The hydrolysate was chilled to 4°, adjusted to pH 1·5 with perchloric acid, and centrifuged. The hydrolyzed RNA in the supernatant was then determined spectrophotometrically.

Synthetase activity was assayed by aminoacylation of tRNA with radioactive amino acids. Unless specifically stated otherwise, the reaction was carried out as follows: Incubation was at 37° in 1 ml containing 100 μmole HEPES buffer (pH 7·5 for pea synthetase, pH 8·0 for soybean synthetase), 1 μmole ATP, 5 μmole $MgCl_2$, tRNA (1–3 mg yeast sRNA, or 0·025–0·25 mg soybean or pea tRNA), 0·01 μmole ^{14}C-amino acid (50 μc/μmole), and 100–500 μg protein. The reaction was monitored by transferring 0·05–0·20 ml samples to ice-cold trichloroacetic acid at times during the assay. The resultant precipitates were collected on glass-fiber discs, washed with three 2-ml washes of cold 0·5% trichloroacetic acid, and two 2-ml washes with Hokin's reagent (935 ml ethanol, 60 ml HOAc, 1·6 ml 10 N NaOH). The filter discs were then dried under heat lamps, placed in vials containing 5 ml scintillation solution (0·3 g dimethyl POPOP and 5 g PPO/l. toluene) and counted in a scintillation spectrometer.

Pyrophosphatase, ATPase, -CCA terminal pyrophosphorylase, and ribonuclease were assayed in the synthetase preparation. The hydrolysis of ATP and inorganic pyrophosphate was determined by measuring the appearance or inorganic orthophosphate by the Fiske–Subbarow method.[64] The 1 ml reaction mixture contained 100 μmole HEPES, pH 8·0, 5 μmole/$MgCl_2$, 10–30 μmole sodium pyrophosphate or disodium ATP, and 10–100 μg enzyme protein. The reaction was monitored by transferring 0·2 ml aliquots to 2 ml cold 5% trichloroacetic acid, centrifuging, and determining the orthophosphate content of the supernatant. Terminal pyrophosphorylase was assayed according to Hurwitz and Furth.[54] The 1 ml reaction mixture contained 100 μmoleHEPES, pH 8·5, 10 μmole/$MgCl_2$, 2 mole/dithiothreitol, 2 mg yeast sRNA, and 0·1 μmole ^{14}C-ATP (10·7 mc/mμmole) with and without 1 μmole CTP and 200–500 μg enzyme protein. Monitoring the reaction, washing the precipitate, and counting procedures were the same as in the charging assay. Ribonuclease activity in the synthetase preparation was measured using ^{14}C- or ^{3}H-RNA as substrate under the conditions of the synthetase assay. The techniques of monitering the reaction (following loss of precipitable counts) were identical to those of the synthetase assay.

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[64] C. FISKE and Y. SUBBAROW, *J. Biol. Chem.* **66**, 375 (1925).