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AMINO ACID SUBSTRATE SPECIFICITY OF ASPARAGINYL-, ASPARTYL- AND GLUTAMINYL-*t*RNA SYNTHETASE ISOLATED FROM HIGHER PLANTS

PETER J LEA and LESLIE FOWDEN*

Department of Botany and Microbiology, University College, Gower Street, London WC1E 6BT

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Key Word Index—*Phaseolus aureus*, *Albizzia julibrissin*, *Vicia sativa*, Leguminosae, amino acid-transfer RNA synthetases, amino acid substrate specificity

Abstract—$AspNH_2$-, Asp- and $GluNH_2$-*t*RNA synthetases were purified from *Phaseolus aureus*, their optimum assay conditions, substrate specificities and salt sensitivities were investigated $AspNH_2$-*t*RNA synthetase from β-cyanoalanine-producing (*Vicia sativa*), and non-producing (*P aureus* and *V faba*) species was able to utilize the analogue as a substrate irrespective of the source of the enzyme Asp-*t*RNA synthetase from *P aureus* was able to utilize α-aminomalonate and *threo*-β-hydroxyAsp as a substrate The transfer of ^{14}C-$GluNH_2$ to *t*RNA, catalyzed by $GluNH_2$-*t*RNA synthetase, was only inhibited by high concentrations of those analogues tested, albizzine was the most efficient, but no difference could be demonstrated between the substrate specificities of the enzyme isolated from an albizzine-producer (*A julibrissin*) and a non-producer (*P aureus*) species

INTRODUCTION

ASPARAGINYL ($AspNH_2$)-, aspartyl (Asp)- and glutaminyl ($GluNH_2$)-*t*RNA synthetases have not previously been investigated in higher plants probably due to the relatively low levels of activity determined in studies on aminoacyl-*t*RNA synthetases [1]

$AspNH_2$-*t*RNA synthetase has been purified from *Lactobacillus arabinosus*,[2] *Escherichia coli*[3] and rabbit liver [4] The enzyme has been shown to synthesize a specific form of $AspNH_2$-*t*RNA which acts as a repressor of $AspNH_2$-synthetase in experimental tumours [5]

Asp-*t*RNA synthetase has been purified from *L arabinosus*[6] and baker's yeast [7,8] The occurrence of multiple forms of the enzyme has been reported in *Neurospora crassa*[9] and *Bacillus brevis*.[10]

$GluNH_2$-*t*RNA synthetase has been purified from *E coli*,[11] yeast, pig liver[12] and rat liver [13] The enzyme is unusual, in that it can only be assayed by the ATP-PPi technique

* Present address Director, Rothamsted Experimental Station, Harpenden, Herts

[1] LEA, P J and NORRIS, R D (1972) *Phytochemistry* **11**, 2897
[2] HEDGCOTH, C, RAVEL, J and SHIVE, W (1963) *Biochem Biophys Res Commun* **13**, 495
[3] MUENCH, K and SAFILLE, P A (1968) *Biochemistry* **7**, 2799
[4] DAVIES, M R and MARSHALL, R D (1972) *Biochem Biophys Res Commun* **47**, 1386
[5] GALLO, R C, LONGMORE, J L and ADAMSON, R H (1970) *Nature* **227**, 1134
[6] NORTON, S J, RAVEL, J M, LEE, C and SHIVE, W (1963) *J Biol Chem* **238**, 269
[7] MOUSTAFA, E and PETERSEN, G (1962) *Nature* **196**, 377
[8] DILLER, R F B and TENER, G M (1971) *Can J Biochem* **49**, 822
[9] BARNETT, W E and EPLER, J L (1966) *Proc Natl Acad Sci U S* **55**, 184
[10] TAITSEVA, G N, ERMOKHINA, T M, SURGUCHEV, A P, SURGUCHEVA, I G and TSENOV, E I (1969) *Biokhimiya* **34**, 325
[11] RAVEL, J M, WANG, S F, HERNEMEYER, C and SHIVE, W (1965) *J Biol Chem* **240**, 432
[12] LEE, L W, RAVEL, J M and SHIVE, W (1967) *Arch Biochem Biophys* **121**, 614
[13] DEUTSCHER, M P (1967) *J Biol Chem* **242**, 1123

when *t*RNA is present in the assay mixture [11–13] Certain species of *Bacillus* may lack a specific $GluNH_2$-*t*RNA synthetase, Glu-*t*RNA^{GluNH_2} has been shown to be formed by Glu-*t*RNA synthetase, which then may be amidated by a $GluNH_2$- or $AspNH_2$-dependent amidotransferase to form $GluNH_2$-*t*RNA^{GluNH_2} [14]

Investigations in the authors' laboratories have provided information about the substrate specificity of a number of higher plant aminoacyl-*t*RNA synthetases [1] We have now purified the above synthetases and determined their general properties, because plants produce a number of non-protein amino acids, which might act as analogue substrates,[15] our studies have placed particular emphasis upon the amino acid substrate specificity of the enzymes The specificities of enzymes from a plant (*Phaseolus aureus*) failing to produce such analogues were compared with those of enzymes isolated from plants producing analogue molecules, e g $AspNH_2$-*t*RNA synthetase from *Vicia sativa* (a producer of β-cyanoalanine, I)[16] and $GluNH_2$-*t*RNA synthetase from *Albizzia julibrissin* (which produces albizziine, II) [17]

$HO_2C\ CH(NH_2)CH_2CN$ (I)

$HO_2C\ CH(NH_2)CH_2NHCONH_2$ (II)

RESULTS

Asparaginyl-tRNA Synthetase

Enzyme Fractionation

$AspNH_2$-*t*RNA synthetase was extracted and purified from 3 plant sources according to the scheme outlined in Table 1 Glycerol (15%, w/v) and mercaptoethanol (25 mM) were present routinely in the extraction buffer, PVP (MW *ca* 44 000, 0 3%, w/v) was only required when extraction was from leaf material, or from *V sativa* seeds which contained high amounts of tannins The precipitation of the enzyme in late ammonium sulphate fractions (60–70% saturation) allowed the removal of a large amount of protein in the earlier fractions The endogenous level of synthetase activity (measured by ATP-PPi exchange) of this fraction was high, but this was considerably reduced by acetone fractionation prior to the preparation being applied to the DEAE-cellulose column

The fractionation scheme employed for the enzyme was essentially the same for each species studied, and each of the three enzymes eluted as a single peak of activity from the DEAE-cellulose column between 110 and 130 ml of gradient buffer In the heterologous acylation experiments it was necessary only to pass a 60–70% saturated ammonium sulphate fraction through a column of Sephadex G75, to remove ribonucleases, *t*RNA and amino acids

Optimum Assay Conditions

ATP-PPi exchange assay The K_m for $AspNH_2$ was calculated as $1\ 8 \times 10^{-4}$ M, a concentration of 5×10^{-3} M was routinely used to determine the V_{max} for particular enzyme fractions The ATP and ^{32}PPi concentrations were maintained at 4 mM, and the optimum Mg^{2+} concentration was determined as 2×10^{-2} M with a K_m of $5\ 8 \times 10^{-3}$ M The pH optimum was 7 8, with Tris-chloride and Tris-acetate being equally effective in promoting

[14] WILCOX, M and NIRENBERG, M (1968) *Proc Natl Acad Sci U S* **61**, 229
[15] FOWDEN, L (1970) in *Progress in Phytochemistry* (REINHOLD, L and LIWSCHITZ, Y , eds), Vol 2, p. 203, Wiley, London
[16] BELL, E A and TIRIMANNA, A S L (1965) *Biochem J* **97**, 104
[17] GMELIN, R , STRAUSS, G and HASENMAIER, G (1958) *Z Naturforsch* **13b**, 252

maximum exchange during 20 min at 40° Putrescine, spermine and spermidine could not substitute for Mg^{2+}, but Mn^{2+} and Co^{2+} stimulated ATP-PPi exchange at 30 and 17%, respectively, of the maximum rate achieved with Mg^{2+}

TABLE 1 PURIFICATION OF $AspNH_2$-*t*RNA SYNTHETASE FROM THREE PLANT SPECIES

Species	Purification treatment	Total protein (mg)	Specific activity*	Relative purification	Contaminating synthetases†
P aureus seed	Homogenate‡	1300	3 4	1	3200
	Ammonium sulphate fraction	192	21 6	6 3	965
	Acetone fraction	42 6	80 2	23 6	243
	DEAE-cellulose	5 2	566	166	18
V faba seed	Homogenate‡	1762	2 8	1	2200
	Ammonium sulphate fraction	225	19 4	6 9	765
	Acetone fraction	47 8	69 3	24 7	192
	DEAE-cellulose	5 9	472	175	21
V sativa seed	Homogenate‡	972	1 9	1	2350
	Ammonium sulphate fraction	142	12 1	6 4	872
	Acetone fraction	37 1	40 1	21 2	176
	DEAE-cellulose	4 1	287	151	24

* Specific activities are expressed as nmol PPi exchanged/min/mg protein
† Expressed as % of $AspNH_2$-*t*RNA synthetase in each fraction
‡ Determined after removal of endogenous amino acids by Sephadex G25

tRNA esterification assay The K_m for $AspNH_2$ was calculated as $1\,2 \times 10^{-4}$ M, but routinely 1 μCi of undiluted ^{3}H-$AspNH_2$ (10 nmol) was used for all assays (final vol 1 ml). The optimum *t*RNA concentration was 0·2 mg/ml; concentrations higher than 1 0 mg/ml were inhibitory. The total level of aminoacylation reached a maximum after 12 min incubation at 37° and remained constant for 60 min The ATP concentration was maintained at 10^{-3} M and the optimum Mg^{2+} concentration determined as $1\,2 \times 10^{-2}$ M with a K_m of $2\,8 \times 10^{-3}$ M The pH optimum was determined as 7 8, with a sharp drop in activity above pH 8 0 Mg^{2+} could be replaced by various divalent cations at 10^{-2} M, activities for Mn^{2+}, Co^{2+}, Ni^{2+} and Ca^{2+} were 72, 45, 15 and 5% respectively of the values obtained with Mg^{2+}. Cd^{2+}, Zn^{2+}, Sn^{2+}, Sr^{2+}, Ba^{2+}, and the three polyamines were unable to catalyze the formation of $AspNH_2$-*t*RNA Cr^{2+} appeared to catalyze a greater formation of ^{3}H-$AspNH_2$-*t*RNA than Mg^{2+}, but this reaction was independent of ATP and was presumed to be due to the formation of a Cr^{2+}-^{3}H-$AspNH_2$ complex

Enzyme Stability

The enzyme could be stored in 40% (w/v) glycerol at −20° for 2 months with less than 10% loss of activity Storage was possible also at 4° in 15% (w/v) glycerol for 48 hr without loss of activity, mercaptoethanol was unnecessary for either of these stabilizations Half the enzyme activity was lost by heating in 0 1 M Tris-chloride buffer pH 7·8 and 15% (w/v) glycerol at 50° for 5 min. The addition of 4 μmol ATP increased the time for 50% loss to 14 min, whilst simultaneous addition of $AspNH_2$ (50 μmol) and ATP increased this time

to 27 min Concentrations of urea above 4 M inactivated the enzyme during incubation at 37° for 5 min, but 70% of the activity could be recovered by dialysis of the extract against 0 1 M Tris-chloride buffer pH 7 8 containing 15% (w/v) glycerol and 25 mM mercaptoethanol *p*-Chloromercuribenzoate (*p*-CMB) inhibited the enzyme (DEAE-cellulose preparation containing 50 μg protein/ml) with a K_i of 5×10^{-5} M Sodium azide at a concentration of 10^{-2} M did not inhibit the enzyme during the assay period

Salt Effects

The enzyme when assayed by ATP-PPi exchange was relatively insensitive to the addition of monovalent ions The concentrations required to give 50% inhibition (50% I), were calculated for Li^+, NH_4^+, Na^+, K^+ and Cs^+ chlorides as 0 7, 0 9, 2 0, 2 5 and 1 3 M, respectively, and values for CNS^-, I^-, Br^-, Cl^-, NO_3^- and $MeCOO^-$ were 0 4, 0 5, 1 2, 2 5, 1 0 and 1 3 M respectively

Aminoacylation of *t*RNA was much more sensitive to the presence of ions, although the relative values of 50% I for the various ions were little changed from those observed in the ATP-PPi exchange assay Plots of activity against ionic strength for NaCl and KCl gave markedly sigmoid curves with a sharp fall in activity between 0 15 and 0 25 M and 0 2 and 0 3 M, respectively Plots of the same data in Debye–Huckel form ($\log_{10}$ activity against $\sqrt{\mu}/1+\sqrt{\mu}$) gave parallel straight lines for values of $\sqrt{\mu}/1+\sqrt{\mu}$ between 0 28 and 0 4 Ammonium chloride inhibited aminoacylation at concentrations above 0 3 M, but increased the rate by 30% at 0 1 M Tris was also inhibitory at concentrations above 0 3 M but had no stimulatory effect at lower concentrations Ethanol (5%, v/v) gave a slight stimulation of activity at low *t*RNA concentrations by apparently lowering the K_m values for the *t*RNA

TABLE 2 KINETIC PARAMETERS OF THE ATP-PPi EXCHANGE STIMULATED BY $AspNH_2$ AND SEVERAL ANALOGUES USING $AspNH_2$-*t*RNA SYNTHETASE FROM *Phaseolus aureus*

Substrate	V_{max}	Concn for V_{max}(M)	K_m
Asparagine	100	5×10^{-3}	$1\,8 \times 10^{-4}$
Isoasparagine	96	5×10^{-3}	$2\,6 \times 10^{-4}$
Aspartate-β-hydroxamate	81	8×10^{-3}	$7\,8 \times 10^{-4}$
β-Cyanoalanine	36	5×10^{-2}	$5\,8 \times 10^{-3}$
threo-β-Hydroxyasparagine	9	*	$4\,2 \times 10^{-2}$
α,γ-Diaminobutyric acid	7	*	$5\,6 \times 10^{-2}$

Results are expressed with respect to the L-form of the amino acid, K_m values are expressed as molar concentrations, V_{max} expressed as a % of the V_{max} for $AspNH_2$

2-Amino-2-carboxyethanesulphonamide, *erythro*-β-hydroxy$AspNH_2$, *O*-phosphoserine, *meso*-diaminosuccinic acid monoamide, 5-diazo-4-oxonorvaline, 5-bromo-4-oxonorvaline, Asp-β-hydrazide, 5-chloro-4-oxonorvaline, β-methyl$AspNH_2$ and *N*-α-methyl$AspNH_2$ failed to promote ATP-PPi exchange at concentrations of 5×10^{-2} M

* Values extrapolated from Lineweaver–Burk plots

Amino Acid Substrate Specificity

ATP-PPi exchange reaction The ability of $AspNH_2$-*t*RNA synthetase from *P aureus* to utilize various analogues is shown in Table 2 Lineweaver–Burk plots for all analogues

tested were linear. None of the analogues promoting ATP-PPi exchange increased the level of exchange when optimum concentrations of $AspNH_2$ were present Only aspartic acid-β-hydroxamate inhibited $AspNH_2$-dependent exchange, when added at a concentration 50-fold in excess of $AspNH_2$.

The hydroxamate itself exhibited abnormal substrate kinetics, the rate of activation progressively decreasing at concentrations above 10^{-2} M, probably due to the nucleophilic inhibition of pyrophosphorolysis by hydroxamates

Aminoacylation of tRNA Transfer of an analogue to *t*RNA was indirectly assessed by measuring the inhibition of ^3H-$AspNH_2$ transfer to *t*RNA produced by various concentrations of unlabelled analogues (Table 3), *t*RNA was always the limiting component in such reaction mixtures Isoasparagine, aspartic acid-β-hydroxamate, β-cyanoalanine and *threo*-β-hydroxyasparagine inhibited the transfer of ^3H-$AspNH_2$ competitively 5-Bromo-4-oxonorvaline at first was thought to inhibit transfer but the percentage inhibition increased with the age and the browning of the analogue solution, little inhibition was detected with freshly prepared solutions and so the inhibitory character of older solutions was attributed to decomposition products, including free bromine

Substrate Specificity of Plants Producing β-Cyanoalanine

β-Cyanoalanine has been shown to occur in certain species of *Vicia*.[16] For these experiments two *Vicia* species were selected, *V faba*, which does not produce β-cyanoalanine and *V. sativa* whose seed contains β-cyanoalanine together with larger amounts of γ-glutamyl-β-cyanoalanine The $AspNH_2$-*t*RNA synthetase from both seeds had very similar properties and both activated β-cyanoalanine in the exchange reaction with K_m values approximately 6×10^{-3} M (K_m for $AspNH_2$, $1\,6 \times 10^{-4}$ M) and V_{max} values of approximately 38%. β-Cyanoalanine (6×10^{-4} M) produced a 50% inhibition of ^3H-$AspNH_2$ (at 10^{-5} M) transfer to *t*RNA The possibility that β-cyanoalanine was being hydrolyzed to $AspNH_2$ either enzymically or chemically during the reaction period was considered, but the substrate potential of β-cyanoalanine did not increase when it was incubated for various periods up to 60 min with synthetase preparations prior to the ATP-PPi exchange assay

TABLE 3 THE EFFECT OF $AspNH_2$ ANALOGUES ON THE TRANSFER OF ^3H-$AspNH_2$ TO *t*RNA USING ENZYME FROM *Phaseolus aureus*

Substrate	Concn required for 50% inhibition (M)	Substrate	Concn required for 50% inhibition (M)
Isoasparagine	7×10^{-5}	β-Cyanoalanine	9×10^{-4}
Aspartate-β-hydroxamate	5×10^{-4}	*threo*-β-Hydroxyasparagine	7×10^{-3}

^3H-$AspNH_2$ present at a concentration of 10^{-5} M

N^4-methyl$AspNH_2$, α,γ-diaminobutyric acid, *S*-carbamoylcysteine, at concentrations of 10^{-2} M, inhibited transfer of ^3H-$AspNH_2$ by 10–15%

2-Amino-2-carboxyethanesulphonamide, *erythro*-β-hydroxy$AspNH_2$, *O*-phosphoserine, Aspdiamide, *meso*-diaminosuccinic acid monoamide, 5-diazo-4-oxonorvaline, 5-bromo-4-oxonorvaline, 5-chloro-4-oxonorvaline, β-methyl$AspNH_2$, Asp-β-hydrazide and *N*-α-methyl$AspNH_2$ failed to inhibit transfer when present at 10^{-2} M concentration

Heterologous Acylation

P aureus seed enzyme was able to acylate *t*RNA from *V sativa* or *V faba* with an efficiency of 83% of that determined for the homologous combination with *P aureus t*RNA *Vicia sativa* and *V faba* enzymes were able to acylate *P aureus t*RNA 68% as efficiency as the *P aureus* enzyme The *t*RNA of *V sativa* and *V faba* appeared to be completely interchangeable, acylation remaining constant when either heterologous combination was tested

Preparations of enzyme and *t*RNA were obtained from the following tissues of *P aureus* (a) dry seed, (b) 5-day-old hypocotyl supernatant fraction, (c) 5-day-old hypocotyl, mitochondrial fraction, and (d) 14-day-old leaf chloroplast fraction No cross-reaction was detected between fractions originating from seed and from mitochondria or chloroplasts The only heterologous acylation reaction established was between seed and hypocotyl supernatant fractions The seed enzyme could catalyze 20% transfer of ^{3}H-AspNH_2 to supernatant *t*RNA, whilst the supernatant enzyme could catalyze a 28% transfer to seed *t*RNA (percentages are relative to the activity measured with the respective homologous combinations)

Aspartyl-tRNA Synthetase

Enzyme Fractionation

Very low level activities of Asp-*t*RNA synthetase were detected in extracts of *P aureus* seed after passage through Sephadex G25 However, a relatively late precipitate obtained during acetone fractionation (at 42–50%, v/v) exhibited high Asp-dependent ATP-PPi exchange This precipitate was either; (a) redissolved in dilute Tris-chloride buffer (0 03 M) and dialyzed against further buffer containing 15% (w/v) glycerol and 25 mM mercaptoethanol, when much inactive protein precipitated and was removed by centrifugation, or (b) redissolved in extraction buffer and subjected to ammonium sulphate fractionation, when a 45–55% saturated fraction was taken and exhaustively dialyzed against 0 03 M Tris-chloride After either of these treatments, the enzyme was purified further on a DEAE-cellulose column The enzyme eluted as a single peak between 85 and 105 ml of elution buffer, the full purification scheme is shown in Table 4

TABLE 4 PURIFICATION OF Asp-*t*RNA SYNTHETASE FROM *Phaseolus aureus* SEED

Purification treatment	Total protein (mg)	Specific activity	Relative purification	Contaminating synthetases‡
Homogenate‡	1300	1 9	1	5750
Acetone fraction	122	18 1	9 5	820
Ammonium sulphate fraction	46	39 2	20 6	260
DEAE-cellulose	4 2	326	171	11

* Specific activities are expressed as nmol PPi exchanged/min/mg protein
† Expressed as a % of Asp-*t*RNA synthetase in each fraction
‡ Determined after removal of endogenous amino acids by Sephadex G25

Optimum Assay Conditions

ATP-PPi exchange The K_m for Asp was found to be $4{\cdot}6 \times 10^{-3}$ M, with 4×10^{-2} M required to obtain the V_{max} The K_m for Mg^{2+} was $6\,9 \times 10^{-3}$ M with an optimum concentration of $1\,2 \times 10^{-2}$ M The optimum pH was 8 2 with a sharp loss of activity below 7 8, but a plateau of high activity was maintained up to a pH of 9 1

Maximum levels of activity were detected by incubating for 15 min at 37° with Tris-chloride buffer Mn^{2+} and Co^{2+} were able to stimulate ATP-PPi exchange, at rates of 35 and 12% of that determined with the optimal concentrations of Mg^{2+}

Aminoacylation of tRNA Although the 40–50% (v/v) acetone fraction gave high values of Asp-dependent ATP-PPi exchange, the fraction even after passage through Sephadex G75 to remove ribonucleases, gave very low levels of transfer of ^{3}H-Asp to *t*RNA, only the DEAE-cellulose eluate catalyzed a significant reaction The optimum *t*RNA concentration was 0 1 mg/ml, and concentrations higher than 0 3 mg/ml were inhibitory The total level of aminoacylation reached a maximum after 10 min incubation at 37° and began to decrease after 15 min assay time The ATP concentration was maintained at 10^{-3} M and the optimum Mg^{2+} concentration was 5×10^{-3} M with a K_m of $1\,1 \times 10^{-3}$ M Mg^{2+} could be replaced by Mn^{2+} and Co^{2+} at 10^{-2} M, with retention of 56 and 34% activity, respectively. Spermine, spermidine and putrescine were able to substitute for Mg^{2+} with efficiencies of 32, 16 and 27% respectively

Enzyme Stability

The enzyme could be stored in a dry form (e g as an acetone precipitate) for 1 month without loss of activity However, in solution even in the presence of 40% (w/v) glycerol, and reagents containing stabilizing –SH groups, 50% of the activity was lost in 72 hr at −20° A loss of 50% activity occurred when the enzyme was heated at 50° for 7 min, the half-life of the enzyme at 50° in the presence of ATP (4 μmol) alone and of ATP (4 μmol) and Asp (100 μmol) together, was increased to 12 and 23 min, respectively

Concentrations of urea above 4 M caused enzyme denaturation in 5 min at 37°, and only 30% of the activity was recovered after dialysis. *p*-CMB inhibited the enzyme and a K_i was determined for the DEAE-cellulose purified enzyme (protein concentration 30 μg/ml) of 4×10^{-5} M Sodium azide (10^{-2} M) did not inhibit the enzyme

Salt Effects

The enzyme, when assayed by ATP-PPi exchange, was inhibited by most monovalent ions Concentrations of Li^+, NH_4^+, Na^+, K^+ and Cs^+ (as chlorides) producing 50% inhibition were 0 6, 0 9, 1·4, 2 3 and 1 1 M respectively, the similar concentrations for IO_3^-, ClO_3^-, CNS^-, I^-, Br^-, Cl^-, NO_3^- and $MeCOO^-$, were 0 04, 0 1, 0 1, 0 1, 0·7, 2 2, 0 7 and 2·0 M, respectively The aminoacylation of *t*RNA was very sensitive to the addition of ions, 50% inhibition values for Li^+, NH_4^+, Na^+, K^+ and Cs^+ were determined as 0·11, 0 14, 0 18, 0 22 and 0 16 M, respectively

Amino Acid Substrate Specificity

ATP-PPi exchange reaction. The ability of Asp-*t*RNA from *P aureus* to utilize various analogues is shown in Table 5 Lineweaver–Burk plots for the substrates were linear No inhibition of Asp-dependent ATP-PPi exchange was detected for the analogues tested

Aminoacyl transfer to tRNA The possibility of transfer of an analogue to *t*RNA was measured indirectly (cf $AspNH_2$-synthetase) by studying the inhibition of transfer of ^{3}H-Asp caused by various concentrations of analogues (Table 6)

TABLE 5 KINETIC PARAMETERS OF THE ATP-PPi EXCHANGE STIMULATED BY Asp AND SEVERAL ANALOGUES USING Asp-*t*RNA SYNTHETASE FROM *Phaseolus aureus* SEED

Substrate	V_{max}	Concn for V_{max} (M)	K_m
Aspartic acid	100	4×10^{-2}	4.6×10^{-3}
α-Aminomalonic acid	105	8×10^{-2}	7.2×10^{-3}
threo-β-Hydroxyaspartic acid	78	10^{-1}	2.4×10^{-2}

Results are expressed with respect to the L-form of the amino acid, K_m values are expressed as molar concentrations, V_{max} expressed as a % of the V_{max} for Asp *threo*-β-MethylAsp, *erythro*-β-hydroxyAsp, *O*-phosphoserine, *O*-methylserine, homoserine, β-cyanoalanine, isoasparagine, *N*-acetylAsp, 5-bromo-4-oxonorvaline and 5-chloro-4-oxonorvaline failed to stimulate ATP-PPi exchange at 5×10^{-2} M

The Dependence of Asp-Stimulated ATP-PPi Exchange upon Asp-tRNA Synthetase

The low transfer of ^{3}H-Asp to *t*RNA effected by an enzyme fraction stimulating a high Asp-dependent ATP-PPi exchange might indicate that the preparation contained more than one enzyme able to catalyze ATP-PPi exchange Asparagine synthetase and arginosuccinate synthetase are other enzymes that are able to catalyze this reaction However, when purified Asp-*t*RNA synthetase was incubated with ^{3}H-Asp, ATP, Mg^{2+} and (a) ammonia, (b) glutamine, or (c) citrulline, neither ^{3}H-$AspNH_2$ from (a) or (b) nor ^{3}H-arginosuccinate (from c) were detected after PC separations

TABLE 6 THE EFFECT OF Asp ANALOGUES ON THE TRANSFER OF ^{3}H-Asp TO *t*RNA USING ENZYME FROM *Phaseolus aureus*

Substrate	Concn required for 50% inhibition (M)	Substrate	Concn required for 50% inhibition (M)
α-Aminomalonic acid	6×10^{-5}	*threo*-β-Methylaspartic acid	4×10^{-3}
threo-β-Hydroxyaspartic acid	8×10^{-4}	*erythro*-β-Hydroxyaspartic acid	1×10^{-2}

^{3}H-Asp present at a concentration of 10^{-5} M
O-phosphoserine, β-cyanoalanine, homoserine, *O*-methylserine, *N*-acetylAsp, 5-bromo-4-oxonorvaline and 5-chloro-4-oxonorvaline failed to inhibit transfer of ^{3}H-Asp to *t*RNA

Glutaminyl-tRNA Synthetase

Enzyme Fractionation

Low or zero activities assayed by ATP-PPi exchange were determined in crude preparations of *P aureus* seed after passage through Sephadex G25 However, the formation of ^{14}C-$GluNH_2$-*t*RNA was catalyzed by crude preparations, after passage through Sephadex

G75 to remove ribonucleases Active preparations of $GluNH_2$-*t*RNA synthetase were obtained by employing the following fractionation procedure (a) precipitate as 50–60% saturated ammonium sulphate fraction, (b) precipitations between 40 and 50% (v/v) acetone, and (c) passage through Sephadex G75 to remove low MW compounds Enzyme was also prepared from cotyledons of *A julibrissin* seedlings (radicles 1 cm in length) by the same purification procedure as that described for *P. aureus* (Table 7)

TABLE 7 PURIFICATION OF $GluNH_2$-*t*RNA SYNTHETASE FROM *Phaseolus aureus* AND *Albizzia julibrissin*

Plant	Purification treatment	Total protein (mg)	Specific activity*	Relative purification
P aureus	Homogenate†	1300	2 1	1
	Ammonium sulphate fraction	226	10 8	5 1
	Acetone fraction	32	62 4	29 6
A julibrissin	Homogenate†	946	1 3	1
	Ammonium sulphate fraction	152	8 1	6 2
	Acetone fraction	26	45 1	34 7

* Specific activity expressed as pmol ^{14}C-$GluNH_2$ transferred to *t*RNA/min/mg protein
† Determined after removal of endogenous amino acids and RNAase by Sephadex G75

Evidence for the Formation of ^{14}C-$GluNH_2$-tRNA

Wilcox and Nirenberg[14] (see Introduction) have indicated that $GluNH_2$ is not transferred directly to *t*RNA. It was therefore important to show in these experiments that the $GluNH_2$ was transferred directly and that, $GluNH_2$-*t*RNA was not derived by amidation of Glu-*t*RNA The transfer of ^{14}C-$GluNH_2$ (10^{-5} M) was 50% inhibited by the addition of 10^{-5} M ^{12}C-$GluNH_2$ when *t*RNA was limiting In contrast the formation of $GluNH_2$-*t*RNA was not inhibited by the same concentration of Glu, although slight inhibition was produced by concentrations of 10^{-3} M No Glu-*t*RNA synthetase activity could be detected in the enzyme preparation used but it catalyzed some conversion of ^{14}C-Glu to ^{14}C-$GluNH_2$, the conversion of ^{14}C-$GluNH_2$ to ^{14}C-Glu did not occur under the same conditions These observations are consistent with the direct transfer of $GluNH_2$ to *t*RNA

Optimum Assay Conditions

ATP-PPi exchange No $GluNH_2$-dependent ATP-PPi activity could be detected with the purified extracts from *P aureus* (although slight exchange was detected with cruder preparations) However, there was evidence of the stimulation of a $GluNH_2$-dependent ATP-PPi exchange by the addition of *t*RNA at pH 7 4 This stimulation (which was complicated by the adsorption of ^{32}PPi on to the *t*RNA, giving higher endogenous counts) never amounted to more than a 200% increase above the endogenous level of ATP-PPi exchange, which was insufficient for accurate study of the enzyme's properties

Aminoacylation of tRNA A concentration of 4×10^{-6} M (0 2 μCi) $GluNH_2$ was routinely used for all assays The optimum *t*RNA concentration was 0·15 mg/ml; concentrations higher than 0·6 mg/ml were inhibitory The total level of aminoacylation reached a maximum after 8 min incubation at 37°, and began to decrease after 12 min ATP was present at 10^{-3} M, and the optimum Mg^{2+} concentration was $2\,5 \times 10^{-2}$ M (K_m $8\,9 \times 10^{-3}$ M) The pH

activity curve showed a sharp peak at 7 8 Only Mn^{2+} among the divalent cations tested could replace Mg^{2+} at 10^{-1} M (42% of maximum activity with Mg^{2+}) Spermine, putrescine and spermidine (all at 10^{-1} M) could also partially substitute for Mg^{2+} (comparable activities were 35, 32 and 12%, respectively) The transfer of ^{14}C-GluNH_2 to *t*RNA was inhibited by salts, the concentrations required for 50% inhibition by sodium salts of Cl^-, Br^- and I^- were 0 2, 0 1 and 0 06 M respectively

TABLE 8 THE EFFECT OF GluNH_2 ANALOGUES ON THE TRANSFER OF ^{14}C-GluNH_2 TO *t*RNA USING ENZYME FROM *Phaseolus aureus* AND *Albizzia julibrissin*

Substrate	Concn required for 50% inhibition (M) *P aureus*	*A julibrissin*
γ-Methyleneglutamine	$7\,1 \times 10^{-3}$	$1\,6 \times 10^{-2}$
Theanine	$5\,6 \times 10^{-3}$	$1\,2 \times 10^{-3}$
S-Carbamoylcysteine	$2\,2 \times 10^{-3}$	$6\,4 \times 10^{-3}$
O-Carbamoylserine	$1\,1 \times 10^{-3}$	$3\,1 \times 10^{-3}$
Albizziine	$8\,1 \times 10^{-4}$	$9\,2 \times 10^{-4}$
Glutamic acid γ-hydroxamate	$3\,8 \times 10^{-2}$	$4\,1 \times 10^{-2}$

^{14}C-GluNH_2 present at a concentration of 4×10^{-6} M
Glutamic acid diamide gave 2–5% inhibition at 4×10^{-2} M for both enzymes

Amino Acid Substrate Specificity

The low rates of ATP-PPi exchange activity prevented the use of this technique in studies on amino acid substrate specificity The possibility that several analogues of GluNH_2 could be utilized by the enzyme was therefore investigated by the indirect method, i e by measuring the inhibitory action upon the transfer of ^{14}C-GluNH_2 to *t*RNA The concentrations of the analogues required to produce 50% inhibition of transfer in systems employing enzymes from *P aureus* and *A julibrissin* are shown in Table 8

DISCUSSION

AspNH_2-tRNA Synthetase

The level of contaminating synthetases (assayed by ATP-PPi exchange) was reduced to a level (≏20%) that would not interfere with substrate specificity studies carried out by the exchange assay The transfer of ^{3}H-AspNH_2 to *t*RNA was not inhibited by the addition of a mixture containing the other normal 19 amino acid constituents of protein, and is therefore presumed to be catalyzed by one specific enzyme The enzyme was stable in the cold, but rather readily deactivated by heating, and partially protected against heat denaturation by ATP and AspNH_2, the protection of aminoacyl-*t*RNA synthetases against heat has been discussed by Norris and Fowden [18,19] The enzyme was denatured by 4 M urea indicating that hydrogen bonding plays an essential part in retaining the enzyme's conformation, and also by low concentrations of *p*-CMB indicating that –SH groups are present at or near the active site of the enzyme The ability of Mn^{2+}, Co^{2+} or Ni^{2+} to replace Mg^{2+} in the

[18] NORRIS, R D and FOWDEN, L (1972) *Phytochemistry* **11**, 2921
[19] NORRIS, R D and FOWDEN, L (1973) *Biochim Biophys Acta* in press

aminoacylation reaction has been well documented [20] However, the failure of the three polyamines tested to substitute partially for Mg^{2+} is unusual

The hierarchy of salt inhibition for the ATP-PPi exchange reaction, $Li^+ > NH_4^+ > Cs^+ > Na^+ > K^+$ and $I^- > Br^- > Cl^-$ is similar to that shown for the Pro-*t*RNA synthetase from *P aureus* [18] The resistance of the ATP-PPi reaction to high salt concentration indicates that the binding of the ATP and amino acid to the enzyme is not by ionic forces The sensitivity of the aminoacylation reaction to salts has frequently been reported in animals and micro-organisms,[21] and may be due to (a) binding of *t*RNA to the enzyme by ionic bonds, or (b) conformational changes in the structure of the *t*RNA molecule The fit of the data to the Debye–Huckel equation would indicate that *t*RNA is bound to the enzyme by simple interacting charges However, the Debye–Huckel theory is only applicable to ionic strengths up to 0 1 M, and so the slopes of this type of graph should be used with caution to estimate the number of charges involved in this type of reaction [21] It has been suggested that the large differences in aminoacylation rate occurring over a narrow range of salt concentrations could form the basis of an *in vivo* mechanism for controlling protein synthesis [22]

The ability of low concentrations of various ions to stimulate the aminoacylation of *t*RNA has been reported previously,[22,23] but the large increases in aminoacylation caused by organic solvents in other systems[24] could not be detected with the $AspNH_2$ enzyme

The most interesting result in substrate specificity studies was the lack of inhibition by aspartic acid diamide The amide is usually a potent inhibitor of aminoacyl-*t*RNA synthetases with a K_i value close to the K_m of the corresponding amino acid [18,26] This result would indicate that the carboxyl group of the amino acid is essential for binding to the enzyme Presumably when isoasparagine acts as a substrate, the molecule is positioned in such a manner that the β-carboxyl group and α-carbamoyl group are able to bind to the active site in a fashion similar to that of $AspNH_2$ Asp-β-hydroxamate was found to stimulate ATP-PPi exchange in *Lactobacillus*,[27] and so the N^4-hydroxyl group clearly does not prevent binding to the enzyme The ability of the *threo* isomer (in contrast to the *erythro* form) of β-hydroxy-$AspNH_2$ to act as an inhibitor is probably due to steric hindrance of the binding of the α-amino group by the *threo* hydroxyl None of the derivatives of 4-oxonorvaline, which are potent inhibitors of $AspNH_2$ synthesis in tumour cells,[28] acted as substrates or inhibitors of the enzyme, clearly being too large to be accommodated at the active site of the enzyme

An investigation into the properties and substrate specificity of the enzyme from 2 species of *Vicia* showed that no significant differences occurred between β-cyanoalanine producing and non-producing species As no direct incorporation into *t*RNA has been measured it is possible that the analogue only inhibits the formation of $AspNH_2$-*t*RNA and is not itself incorporated into protein Furthermore the endogenous concentration of β-cyanoalanine in the seed of *V. sativa* may be insufficient in comparison with that of $AspNH_2$ (which has a K_m value 30 times smaller than β-cyanoalanine) to effect any significant competition,

[20] MEHLER, A H and CHAKRABURTY, K (1972) in *Advances in Enzymology* (MEISTER, A , ed), Vol 35, p 443, Interscience, New York
[21] LOFTFIELD, R B (1971) in *Protein Synthesis* (MCCONKEY, E , ed), Vol 1, p 1, Marcel Dekker, New York
[22] SMITH, D W E (1969) *J Biol Chem* **244,** 896
[23] YU, C T and HIRSH, D (1967) *Biochim Biophys Acta* **142,** 149
[24] LOFTFIELD, R B and EIGNER, E. A (1967) *J Biol Chem* **242,** 5355
[25] RITTER, P O , HULL, F J and JACKOBSON, K B (1969) *Biochim Biophys Acta* **179,** 524
[26] PAPAS, T S and MEHLER, A H (1970) *J Biol Chem* **245,** 1588
[27] NORTON, S J. and CHEN, Y T (1969) *Arch Biochem Biophys* **129,** 560
[28] CHOU, T C and HANDSCHUMACHER, R E (1972) *Biochem Pharmacol* **21,** 39

either as a substrate or as an inhibitor If this were so, then no evolutionary pressure would exist in *V sativa* leading to a modification of the substrate specificity of its $AspNH_2$-*t*RNA synthetase It is also intriguing to realize that even if β-cyanoalanine was incorporated into the plant's proteins, such residues might be converted into $AspNH_2$ moieties by subsequent nitrilase action

There have been conflicting reports concerning the ability of a number of cytoplasmic, mitochondrial and chloroplastic synthetases to cross-react with their heterologous *t*RNAs [1 29] A further more detailed study into the multiplicity of $tRNA^{AspNH_2}$ in plants will be carried out

Aspartyl-tRNA Synthetase

The degree of purity of this synthetase obtained from *P aureus* was similar to that of the $AspNH_2$ enzyme The idea that Asp-dependent ATP-PPi exchange catalyzed by the enzyme preparation was due to contaminating $AspNH_2$ synthetase or arginosuccinate synthetase activity was disproved, and no $AspNH_2$- or Glu-dependent ATP-PPi exchange could be detected in the purified enzyme fraction The transfer of ^{3}H-Asp to *t*RNA was not inhibited by a mixture of the other 19 protein amino acids The low level of aminoacylation observed may be due to the presence of an inhibitor in the enzyme and/or *t*RNA preparations or possibly to a low level of $tRNA^{Asp}$ in the extracted *t*RNA

The effect of 4 M urea, *p*-CMB and sodium azide were similar to that demonstrated for the $AspNH_2$ enzyme Mn^{2+} and Co^{2+} were again able to substitute for Mg^{2+}, but with this enzyme the three polyamines were also able to substitute (cf results with *E coli* aminoacyl-*t*RNA synthetases) [30]

The ATP-PPi exchange reaction was a little more sensitive to salt than that catalyzed by the $AspNH_2$ enzyme, however, the degree of salt inhibition was not comparable with the extreme sensitivity by Glu-*t*RNA synthetase from *P aureus* [31] The aminoacylation reaction showed the same high sensitivity to salts as that described for the $AspNH_2$ enzyme

α-Aminomalonic acid has been shown to act as an Asp analogue in the following enzyme systems (a) Glu-dependent $AspNH_2$ synthetase,[32] (b) Asp-decarboxylase,[33] and (c) Asp-transcarbamylase [34] The ability of the Asp-*t*RNA synthetase to recognize this lower homologue of Asp has parallels in the binding of smaller analogue molecules to other plant aminoacyl-*t*RNA synthetases, e g 2-amino-4-methylhex-4-enoic acid by Phe-*t*RNA synthetase[35] and azetidine-2-carboxylic acid by Pro-*t*RNA synthetase [18] The observation that the *threo* stereoisomer, but not the *erythro* form of β-hydroxyAsp acts as an analogue for Asp is in agreement with observations with other enzymes, e g arginosuccinate synthetase,[36] Asp-decarboxylase,[33] and $GluNH_2$-dependent $AspNH_2$ synthetase [32] However, the stereospecificity is reversed when β-hydroxyAsp isomers are tested with Asp-transcarbylamase[34] and Asp-aminotransferase [37] Irrespective of the stereoisomer of β-hydroxyAsp utilized by an enzyme the corresponding β-methyl form is usually a poorer substrate,[32 34] although this is not so for arginosuccinate synthetase [36] It is possible that with the enzyme under study the

[29] BURKARD, G , GUILLEMAUT, P and WEIL, J H (1970) *Biochim Biophys Acta* **224,** 184
[30] TAKEDA, Y and IGARASHI, K (1969) *Biochem Biophys Res Commun* **37,** 917
[31] LEA, P J and FOWDEN, L (1972) *Phytochemistry* **11,** 2129
[32] HOROWITZ, B and MEISTER, A (1972) *J, Biol Chem* **247,** 6708
[33] TATE, S S , and MEISTER, A (1970) in *Advances in Enzymology* (MEISTER, A , ed), Vol 35, p 503, Interscience, New York
[34] DAVIES, G E , VANAMAN, T C and STARK, G (1970) *J Biol Chem* **245,** 1175
[35] ANDERSON, J W and FOWDEN, L (1970) *Biochem J* **116,** 677
[36] RACHOVANSKY, O and RATNER, S (1968) *Arch Biochem Biophys* **127,** 688
[37] JENKINS, W T (1961) *J Biol Chem* **236,** 1121

erythro-hydroxyl group would interfere with the attachment of the α-amino group to the active site, the *threo* group being on the other side of the molecule would not exert such an effect

Glutaminyl-tRNA Synthetase

The lack of ATP-PPi exchange catalyzed by $GluNH_2$-*t*RNA synthetase in the absence of *t*RNA is in agreement with the properties reported for this enzyme from other sources [11–13] The low $GluNH_2$-dependent ATP-PPi exchange demonstrated in the presence of *t*RNA could be due to an inhibitor in the *t*RNA preparation or to the lack of an active specific $tRNA^{GluNH_2}$, *t*RNA probably behaves as an activator for the enzyme in the ATP-PPi exchange reaction and not as an acceptor of the aminoacyl group [38] However, the *P aureus* enzyme was able to catalyze the formation of ^{14}C-$GluNH_2$-*t*RNA; this reaction was inhibited by the addition of unlabelled $GluNH_2$ or of high concentrations of Glu The enzyme probably contained low glutamine synthetase activity producing small amounts of inhibitory $GluNH_2$ when Glu was added at high concentrations

The optimum Mg^{2+} ATP ratio for the aminoacylation of *t*RNA was 25 1 for the $GluNH_2$ enzyme, 12 5 1 for the $AspNH_2$ enzyme, and 5 1 for the Asp enzyme It is clear that the Mg^{2+} ATP ratio must be varied during initial experiments designed to determine the optimum conditions for a particular aminoacylation reaction

The enzyme was more specific than either the $AspNH_2$ or the Asp enzyme in its use of divalent cations in place of Mg^{2+}, but was able to utilize all three polyamines The extent of inhibition of aminoacylation by low concentrations of salts was similar to that shown by the other two enzymes, and so again the activity of an enzyme concerned in the initial step in protein synthesis was quite susceptible to small changes in salt concentrations

None of the analogues tested acted as potent inhibitors of the transfer of ^{14}C-$GluNH_2$ to *t*RNA; for instance at least a 200-fold excess of albizziine was required to produce a 50% inhibition of aminoacylation There was little difference between the enzymes isolated from *P aureus* and *A julibrissin* (a natural producer of albizziine), although the *A. julibrissin* enzyme required slightly higher concenttations of analogues to produce 50% inhibition

Although the seed of *A julibrissin* contains a substantial concentration of albizziine, the transfer of significant amounts of the analogue to RNA or into protein seems unlikely, and probably it has little effect upon the formation of $GluNH_2$-*t*RNA It is possible that within the cell there is compartmentation which prevents the access of albizziine to the ribosomes

The negligible inhibition by Glu-diamide again indicates the possible involvement of the α-carboxyl group in the binding of the amino acid to the enzyme The presence of a C–N–C bond (albizziine), a C–O–C bond (*O*-carbamoylserine), or a C–S–C bond (*S*-carbamoylcysteine), instead of the C–C–C bond of $GluNH_2$ alters the distance between the β-carbon and the amide carbon atom from 3 08 Å in $GluNH_2$, to 2 94, 2 86 and 3 62 Å, respectively, in three analogues [39] The inhibitory action of these analogues (albizziine > *O*-carbamoylserine > *S*-carbamoylcysteine) clearly reflects these differences in molecular size Similarly, structural alterations that increase the size of the amide group, e g the introduction of a hydroxamate or an N^5-ethyl substituent (theanine), have a marked influence on enzyme binding The relative inertness of γ-methyleneglutamine as a $GluNH_2$ analogue may be compared with the inability of γ-methyleneglutamic acid to act as a substrate for the Glu-*t*RNA synthetase from *P aureus* [31]

[38] MITRA, S K and MEHLER, A. H (1967) *J Biol Chem* **242**, 5490
[39] PAULING, L (1948) in *The Nature of the Chemical Bond*, Cornell University Press, New York

EXPERIMENTAL

Plant materials Seeds of *A julibrissin* were a gift from Dr M Mazelis, Davis, California, U S A Seeds of *P aureus*, *V sativa* and *V faba* were obtained commercially

Amino acids All samples were tested for purity by PC The L-isomers of the protein amino acids were high purity commercial samples Other amino acids were obtained as follows L-*threo*-β-MethylAsp was a gift from Dr H J Bright, Pennsylvania, U S A DL-*threo*-β-HydroxyAsp was prepared by reducing the *N*-benzyl derivative, a gift from Dr C H Stammer, Georgia, U S A DL-*erythro*-β-HydroxyAsp was a gift from Professor J G Morris, Aberystwyth *Meso*-diaminosuccinic acid monoamide 2HBr, 5-diazo-4-oxo-L-norvaline, 5-bromo-4-oxo-L-norvaline, 5-chloro-4-oxo-L-norvaline HCl and DL-β-methylAspNH$_2$ were gifts from Professor R E Handschumacher, Connecticut, U S A L-Asp-β-hydrazide, *S*-carbamoyl-L-cysteine and *N*-α-methyl-DL-AspNH$_2$ were gifts from Dr I Chibata, Tanabe Seiyaku Co Ltd , Osaka, Japan L-*erythro*-β-HydroxyAspNH$_2$ and L-*threo*-β-hydroxyAspNH$_2$ were gifts from Dr A Singerman, Jerusalem IsoAspNH$_2$ was a gift from Dr G L Tritsch, New York, U S A L-Glu-diamide was a gift from Professor F C Steward, Cornell, U S A Theanine was a gift from Dr K Sasaoka, Kyoto, Japan L-γ-MethyleneGluNH$_2$ (*Arachis hypogea*) and L-albizziine (*Acacia armata*) were natural isolates The potassium salt of α-aminomalonic acid and L-*N*-methylAspNH$_2$ were synthesized in our laboratory DL-*O*-Phosphoserine (Koch–Light & Co Ltd), L-2-amino-2-carboxyethanesulphonamide (Ciba, New Jersey, U S A) L-β-cyanoalanine and L-α,γ-diaminobutyric acid (Calbiochem, U S A), DL-*N*-acetylAsp, DL-β-Asphydroxamate and L-γ-Gluhydroxamate (Sigma & Co Ltd), were obtained commercially

Radioisotopic chemicals L-[G-^{3}H]Asparagine (185 mCi/mmol), L-[G-^{3}H]aspartic acid (220 mCi/mmol), L-[U-^{14}C]glutamic acid (225 mCi/mmol), L-[U-^{14}C]glutamine (50 mCi/mmol) and sodium [^{32}P]orthophosphate were obtained from the Radiochemical Centre, Amersham

Enzyme fractionation All operations were carried out in a cold room at 1–4° Seed meal was ground manually with extraction medium (0 1 M Tris-chloride buffer pH 7 8, containing 15% (w/v) glycerol, 20 mM $MgCl_2$ and 25 mM mercaptoethanol), 10 ml/g seed, and the extract subjected to acetone and ammonium sulphate fractionation as described in an earlier paper [31] The precise fractions taken are described in the text, for each of the enzymes under investigation Sephadex G75 chromatography was carried out on columns equilibrated with the extraction medium at a strength of 0 05 M Tris DEAE-cellulose chromatography was carried out on a column (32 × 1 7 cm dia) equilibrated with 0 03 M Tris-chloride buffer, pH 7 8, containing 15% (w/v) glycerol and 25 mM mercaptoethanol, and developed with a linear gradient of 0–0 4 M KCl (200 ml in each gradient vessel) Chloroplasts were isolated by the method described by Leech[40] and purified on a sucrose gradient The enzymes were solubilized by stirring the chloroplasts in 0 1% Triton X100 for 30 min and centrifuging off the chloroplast membranes at 15 000 *g* for 15 min Mitochondria were prepared by the method described by Bonner,[41] and washed twice to remove soluble enzymes Mitochondrial enzymes were solubilized in 0 1% Triton X100 followed by centrifugation at 30 000 *g* for 30 min

tRNA Isolation The method of Vanderhoef *et al* [42] was employed to extract *t*RNA from the chloroplasts, mitochondria and supernatant extracts Seed meal was extracted by the same method but double the suggested volume of Tris-chloride buffer was used in the initial extraction Preparations had E_{260} E_{280} ratios >2 0 and E_{260} E_{230} ratios >1 8 before use in enzyme assays

Protein determination The method of Lowry *et al* [43] was normally adopted, using bovine serum albumin as a standard Fractions eluted from DEAE-cellulose columns were assayed by determining absorbance at 260 and 280 nm by the method described by Layne [44]

Assay Procedures (a) *ATP-PPi exchange system* The method was based on that described by DeMoss and Novelli [45] The precise assay mixtures are discussed for each individual enzyme in the text The exchange rates were calculated by the method of Davie *et al* [46] (b) *Aminoacyl-tRNA formation* The method followed was that described by Vanderhoef *et al* ,[42] precipitates were collected on glass fibre discs and radioactivity determined by scintillation counting Individual assay mixtures are discussed in the text

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[40] LEECH, R M (1963) *Biochim Biophys Acta* **71**, 253
[41] BONNER, W D (1967) in *Methods in Enzymology* (ESTABROOK, R W and PULLMAN, M , eds), Vol 10, p 126, Academic Press, New York
[42] VANDERHOEF, L N , BOHANNON, R F and KEY, J L (1970) *Phytochemistry* **9**, 2291
[43] LOWRY, O H , ROSEBROUGH, N J , FARR, A L and RANDALL, R J (1951) *J Biol Chem* **193**, 265
[44] LAYNE, E (1957) in *Methods in Enzymology* (COLOWICK, S P and KAPLAN, N O , eds), Vol 3, p 447, Academic Press, New York
[45] DEMOSS, J A and NOVELLI, G D (1956) *Biochim Biophys Acta* **22**, 49
[46] DAVIE, E W , KONINGSBERGER, V V and LIPMANN, F (1956) *Arch Biochem Biophys* **65**, 21