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Properties and Substrate Specificities of the Phenylalanyl-Transfer-Ribonucleic Acid Synthetases of *Aesculus* Species

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1. Phenylalanyl-tRNA synthetases have been partially purified from cotyledons of seeds of *Aesculus californica*, which contains 2-amino-4-methylhex-4-enoic acid, and from four other species of *Aesculus* that do not contain this amino acid. The *A. californica* preparation was free from other aminoacyl-tRNA synthetases, and the contaminating synthetase activity in preparations from *A. hippocastanum* was decreased to acceptable limits by conducting assays of pyrophosphate exchange activity in 0.5M-potassium chloride. 2. The phenylalanyl-tRNA synthetase from each species activated 2-amino-4-methylhex-4-enoic acid with K_m 30–40 times that for phenylalanine. The maximum velocity for 2-amino-4-methylhex-4-enoic acid was only 30% of that for phenylalanine with the *A. californica* enzyme, but the maximum velocities for the two substrates were identical for the other four species. 3. 2-Amino-4-methylhex-4-enoic acid was not found in the protein of *A. californica*, so discrimination against this amino acid probably occurs in the step of transfer to tRNA, though subcellular localization, or subsequent steps of protein synthesis could be involved. 4. Crotylglycine, methallylglycine, ethallylglycine, 2-aminohex-4,5-dienoic acid, 2-amino-5-methylhex-4-enoic acid, 2-amino-4-methylhex-4-enoic acid, β-(thien-2-yl)alanine, β-(pyrazol-1-yl)alanine, phenylserine and *m*-fluorophenylalanine were substrates for pyrophosphate exchange catalysed by the phenylalanyl-tRNA synthetases of *A. californica* or *A. hippocastanum*. Allylglycine, phenylglycine and 2-amino-4-phenylbutyric acid were inactive.

Seeds of the Californian buckeye (*Aesculus californica*) contain large amounts of AMHA† (about 40μmol/g fresh wt.) representing approx. 50% of the total free amino acids in this tissue (Fowden & Smith, 1968). AMHA inhibits the growth of *Leuconostoc dextranicum* and the inhibition is alleviated by phenylalanine (Edelson, Skinner, Ravel & Shive, 1959), suggesting that AMHA competes with phenylalanine in some essential metabolic process. One reaction in which AMHA might compete with phenylalanine is in the formation of the phenylalanyladenylate catalysed by the enzyme phenylalanyl-tRNA synthetase. This reaction represents the first step in the incorporation of phenylalanine into protein; the phenylalanyl residue is subsequently transferred from the adenylate to give phenylalanyl-tRNA and then it is incorporated into protein by a template-directed mechanism. In preliminary experiments Fowden & Smith (1968) found that AMHA inhibited the growth of seedlings of mung bean (*Phaseolus aureus*); AMHA could also act as a substrate for pyrophosphate exchange catalysed by the phenylalanyl-tRNA synthetase of mung bean (Smith & Fowden, 1968). Since AMHA could not be detected in a protein fraction prepared from seeds of *A. californica* (i.e. not more than one residue of AMHA/1000 residues of protein-bound amino acids) this species must have developed a mechanism for excluding AMHA from its protein molecules. Discrimination against AMHA might occur at the level of amino acid activation, and so a comparative survey of the amino acid substrate specificity of the phenylalanyl-tRNA synthetase preparations from *A. californica* and other *Aesculus* species has been made.

The genus *Aesculus* contains 14 species, which are grouped into five sections (Hardin, 1957, 1960). A systematic survey of the free amino acids present in 11 species representing all five sections has shown that AMHA and related compounds are confined to members of the section Calothyrsus, which

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† Abbreviation: AMHA, 2-amino-4-methylhex-4-enoic acid.

includes *A. californica* (Fowden, Anderson & Smith, 1970). Other species from the different subgeneric sections selected for phenylalanyl-tRNA synthetase preparation were *A. hippocastanum* (section Aesculus), *A. glabra* (section Pavia), *A. parviflora* (section Macrothyrsus) and *A. parryi* (section Parryaneae).

The phenylalanyl-tRNA synthetases from *A. californica* and *A. hippocastanum* were used to determine which members of a range of amino acids, structurally related to phenylalanine, some of which are plant products, could serve as substrates for phenylalanyl-tRNA synthetase.

MATERIALS AND METHODS

Chemicals. L-Phenylalanine (chromatographically pure) was obtained from Mann Research Laboratories Inc., New York, N.Y., U.S.A. DL-AMHA (containing glycine as a trace contaminant) was synthesized as described by Fowden & Smith (1968). ATP (sodium salt) was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; the pH was adjusted to 7.5 with KOH before use. [^{32}P]Orthophosphate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., converted into [^{32}P]pyrophosphate and adjusted to 0.25Ci/mol (Anderson & Fowden, 1969). Protamine sulphate (ex salmon roe) was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., DEAE-cellulose (DE52) from Whatman (W. and R. Balston Ltd., Maidstone, Kent, U.K.) and Sephadex G-50 (coarse grade) from Pharmacia, Uppsala, Sweden.

Analysis of protein of A. californica *for AMHA residues.* Cotyledon and embryo tissue from dried seed of *A. californica* was finely ground and the flour (5g) was extracted twice with 25ml portions of propan-2-ol to remove fatty substances. Protein was extracted from the alcohol-free residue by extracting three times with 25ml of a solution containing borate (0.05M, pH10), KCl (0.05M) and Teepol 410 (1%, v/v) (Wolfe & Fowden, 1957); each extraction was continued for 48h with shaking. The combined extract was adjusted to pH4 with acetic acid and protein was precipitated by heating at 60°C for 30min. The protein was successively washed with 5% (w/v) trichloroacetic acid (90°C, twice), hot water, ethanol at 75°C, acetone and ether. The protein was hydrolysed with 2.5M-$Ba(OH)_2$ for 18h at 100°C; hot mineral acid destroys AMHA. The amino acids present in portions of the hydrolysate representing 5mg of protein were separated on two-dimensional paper chromatograms (Whatman no. 3MM paper) with 75% (w/v) phenol in the presence of NH_3 vapour and butan-1-ol–acetic acid–water (90:10:29, by vol.) as solvents. Other samples of the hydrolysate were hydrogenated in the presence of Adams PtO_2 catalyst (Fowden & Smith, 1968) before chromatography; under these conditions AMHA was reduced to homoisoleucine, which was more effectively separated from other amino acids in the solvent system used. These procedures would enable AMHA to be detected if it were present in the protein in excess of 0.05–0.1%.

Extraction and purification of phenylalanyl-tRNA synthetase from cotyledons of A. californica *seeds.* Fruits

Table 1. *Removal of contaminating aminoacyl-tRNA synthetases from phenylalanyl-tRNA synthetases of* A. californiac *(1967 harvest) and* A. parviflora *by precipitation with acetone*

Species	Fraction	Absolute activity (units)		Specific activity (units/mg of protein)	
		Phenylalanyl-tRNA synthetase	Other aminoacyl-tRNA synthetases	Phenylalanyl-tRNA synthetase	Other aminoacyl-tRNA synthetases
A. californica	0.293–0.342g of $(NH_4)_2SO_4$/ml	7800	22400	15.8	45.4
	0–25% acetone	375	232	14.7	9.1
	25–35% acetone	1530	400	99.3	26.0
	35–45% acetone	62	4470	1.6	114.5
A. parviflora	0.293–0.342g of $(NH_4)_2SO_4$/ml	1050	4100	20.3	58.1
	0–25% acetone	3	7	5.3	6.9
	25–40% acetone	415	160	213.5	82.1
	40–50% acetone	25	200	2.5	20.2
	50–60% acetone	0	0	0	0

Table 2. *Activity of the individual aminoacyl-tRNA synthetases during purification of phenylalanyl-tRNA synthetase of* A. californica (1967 *harvest) and activity of individual aminoacyl-tRNA synthetases in the* 25–35% *acetone fraction prepared from seeds of* A. californica *harvested in* 1968

Results are expressed as percentages of phenylalanine-dependent pyrophosphate exchange.

	1967 harvest				1968 harvest
Substrate	Crude extract	$(NH_4)_2SO_4$ treatment (0.293–0.342 g/ml)	Acetone treatment (25–35%)	DEAE-cellulose treatment (phenylalanyl-tRNA synthetase peak)	Acetone treatment (25–35%)
Alanine	25	3	0	0	0
Arginine	25	5	1	3	0.6
Asparagine	20	3	0	0	0.1
Aspartic acid	32	6	0	0	0
Cysteine	0	2	0	0	0
Glutamic acid	13	3	0	1	0.2
Glutamine	20	5	1	0	0
Glycine	37	6	7	1	0.4
Histidine	48	30	0	0	1.3
Isoleucine	23	21	5	13	1.0
Leucine	39	49	11	17	5.1
Lysine	22	2	0	0	0.5
Methionine	15	2	1	0	0
Phenylalanine	100	100	100	100	100*
Proline	39	0	1	0	0.1
Serine	22	1	2	0	2.2
Threonine	108	51	19	11	3.5
Tryptophan	0	0	0	0	0.2
Tyrosine	43	2	2	0	2.1
Valine	42	35	30	1	0.9
Complete mixture less phenylalanine		170	60	31	
AMHA					34
Endogenous					0.7

* The specific activity of the phenylalanyl-tRNA synthetase was 52.8 units/mg of protein.

were collected in California by Professor E. E. Conn, University of California, Davis, Calif., U.S.A. and forwarded by air mail to London. On arrival, the pericarps were removed and desiccation of the seeds was retarded by storing them in a cold-room at 2°C; the water content of cotyledons from seeds stored for 8 months was approx. 80% of the fresh wt. Seeds used during the first 3 months after harvest tended to yield brown extracts whose enzymic activity was variable. After the seeds had matured for 3 months, pale-yellow extracts of high activity were obtained; such seeds were used as a source of enzyme in all experiments conducted with *A. californica*. The addition of thioglycollate to the extraction medium did not augment the yield of enzyme provided that the maturation period was observed.

Cotyledons were dissected from matured seeds and extracted in a Waring Blendor with 1.56ml of medium-1 (0.1M-tris–HCl buffer, pH7.5, containing 20mM-$MgCl_2$)/g fresh wt. (homogenization time approx. 3min/100g of cotyledons). The extract was squeezed through muslin and the supernatant solution was recovered by centrifugation at 10000*g* for 20min. Solid $(NH_4)_2SO_4$ was added (0.293g/ml of extract) and all material precipitated was discarded. Additional $(NH_4)_2SO_4$ was added (0.049g/ml) and the protein precipitate (which contained phenylalanyl-tRNA synthetase) was sedimented and redissolved in approx. 0.5ml of medium-2 (0.05M-tris–HCl buffer, pH7.5, containing 20mM-$MgCl_2$)/g of original cotyledons. The tyrosyl-tRNA synthetase was not precipitated.

Acetone was slowly added to a final concentration of 25% (v/v) while the temperature was lowered to −5°C. The supernatant was slowly adjusted to 32% (v/v) acetone, and the resulting precipitate was centrifuged, re-extracted with medium 2 and clarified by centrifugation at 10000*g* for 20min. This two-step acetone fractionation was preferable to stepwise fractionation in 5% steps, giving less than 15% contamination by other aminoacyl-tRNA synthetases as judged from the pyrophosphate exchange assay, but for seeds collected in 1968 the fraction precipitated by 25–35% (v/v) acetone gave a preparation essentially free from other aminoacyl-tRNA synthetases (Tables 1 and 2).

Further purification of the preparation from the 1967 batch of seeds was attempted on a DEAE-cellulose column (30cm × 1.5cm) equilibrated with medium-2 and developed with a linear gradient (0–0.2M) of KCl in 200ml of medium-2. The peak of phenylalanyl-tRNA synthetase activity still contained appreciable amounts of isoleucyl-tRNA synthetase, leucyl-tRNA synthetase and threonyl-tRNA synthetase activities (Table 2).

Extraction and purification of the phenylalanyl-tRNA synthetase from the cotyledons of A. hippocastanum *seeds.* Seeds were collected from a single tree in London and stored at 2°C. Cotyledons were dissected and extracted in a Waring Blendor with 3ml of medium-1/g fresh wt. The extract was squeezed through muslin and the supernatant solution was recovered by centrifugation at 10000*g* for 20min. The extract was passed through muslin after centrifugation to remove a copious layer of lipid. A solution containing protamine sulphate (2%, w/v) in medium-1 (1vol.) was added to the crude supernatant solution (5vol.). The precipitated material was washed with medium-3 (0.02M-tris–HCl buffer, pH7.5, containing 20mM-$MgCl_2$) and then extracted with a solution containing 1M-KCl in medium-3 (0.4ml/g of original cotyledons) to solubilize the phenylalanyl-tRNA synthetase. Insoluble material was removed by centrifugation and the supernatant solution was passed through a column of Sephadex G-50 (equilibrated and eluted with medium-3) with 15 bed vol./vol. of supernatant applied. Passage through Sephadex G-50 removed the KCl, retarded protamine sulphate and a yellow pigment, and rendered much inactive material insoluble, whereas the phenylalanyl-tRNA synthetase remained in solution. The most turbid material eluted from the column was collected and insoluble material was removed by centrifugation. Saturated $(NH_4)_2SO_4$ solution (1.5vol., adjusted to pH7.5 with NH_3) was added and the precipitate was redissolved in medium-4 (0.01M-tris–HCl buffer, pH7.5, containing 20mM-$MgCl_2$).

Further purification was achieved by chromatography on a DEAE-cellulose column (30cm × 1.5cm) equilibrated with medium-4 and developed with a linear gradient (0–0.15M) of KCl in 300ml of medium-4. Some leucyl-tRNA synthetase eluted with phenylalanyl-tRNA synthetase and the pyrophosphate exchange of leucyl-tRNA synthetase never exceeded 25% of that effected by the phenylalanyl-tRNA synthetase; some preparations prepared from seed stored at 2°C for 9 months contained negligible amounts of leucyl- and valyl-tRNA synthetases.

Cotyledons of dry seeds (water content approx. 8%) were an equally satisfactory source of phenylalanyl-tRNA synthetase from *A. hippocastanum*. Cotyledons were ground into a fine flour and extracted with 8.6ml of medium-1/g.

Extraction and purification of phenylalanyl-tRNA synthetase from cotyledons of A. glabra *seeds.* Seeds were supplied by the Arnold Arboretum, Jamaica Plain, Mass., U.S.A. and stored at 2°C until required. The enzyme was extracted and purified by the procedure used for the preparation of phenylalanyl-tRNA synthetase from *A. hippocastanum* except that purification on DEAE-cellulose was not attempted.

Extraction and purification of phenylalanyl-tRNA synthetase from cotyledons of A. parviflora *seeds.* Seeds of *A. parviflora f. serotina* were supplied by the Arnold Arboretum and stored at −15°C until required. Cotyledons were extracted in a Waring Blendor with 3ml of medium-1/g fresh wt. The extract was squeezed through muslin and the supernatant solution recovered by centrifugation at 10000*g* for 20min. A solution containing 2% (w/v) of protamine sulphate in medium-1 was added to the crude supernatant (5vol.). The precipitate (containing about 1–2% of the phenylalanyl-tRNA synthetase) was discarded. Solid $(NH_4)_2SO_4$ was added (0.293g/ml of solution) and the precipitate was discarded. Additional $(NH_4)_2SO_4$ was added (0.049g/ml) and the precipitated material was dissolved in 0.67ml of medium-1/g of original cotyledons. The solution was clarified by centrifugation and fractionated with acetone as described for the preparation of phenylalanyl-tRNA synthetase from *A. californica* except that protein was collected between 25 and 40% (v/v) acetone. Precipitated protein was extracted with medium-1 and any insoluble material was removed by a final centrifugation at 10000*g* for 20min.

Extraction and purification of phenylalanyl-tRNA

synthetase from cotyledons of A. parryi *seeds.* Seeds of this species were collected in Baja, California, by Dr R. V. Moran, San Diego, Natural History Museum, Calif., U.S.A. and forwarded by airmail. The enzyme was prepared immediately the seed arrived in London by the procedure used for the preparation of phenylalanyl-tRNA synthetase from *A. parviflora.*

Assay of phenylalanyl-tRNA synthetase and other aminoacyl-tRNA synthetases. Synthetase activity was measured by pyrophosphate exchange. Assay mixtures contained 2μmol of K_2Na_2ATP, 2μmol of sodium [^{32}P]pyrophosphate (0.25μCi/μmol) 14μmol of $MgCl_2$, 1 or 2μmol of the appropriate L-amino acid (sufficient to saturate the appropriate aminoacyl-tRNA synthetase) and 70μmol of tris–HCl buffer, pH 7.5, in 1ml; assays were performed at 35°C for 15min. The phenylalanyl-tRNA synthetases of *A. glabra* and *A. hippocastanum* were never fully separated from leucyl-tRNA synthetase. Assays of phenylalanyl-tRNA synthetases from these species always contained 500μmol of KCl to inhibit leucyl-tRNA synthetase (Anderson & Fowden, 1970). The amount of AMHA used as substrate for the phenylalanyl-tRNA synthetase was usually 10 or 20μmol of the L-isomer (supplied as the racemate). All concentrations of amino acid substrates specified in this paper refer to the L-isomer though if a racemate was used, this is stated. Sometimes an amino acid mixture containing 1μmol of each of the protein amino acids (except cystine and hydroxyproline) was used as substrate; cystine and hydroxyproline were not included as there is no evidence that separate synthetases exist for these compounds (Peterson, 1967). All other procedures of the assay were as described by Anderson & Fowden (1969).

Synthetase activity is expressed as the difference in pyrophosphate exchange in nmol/min (synthetase units) between assays with and without added amino acid(s). Rate of exchange was calculated by the method of Davie, Koningsberger & Lipmann (1956) but usually the enzyme was diluted before assay to decrease the exchange to less than 5% so that the amount of pyrophosphate exchanged was proportional to the amount of label in the ATP.

The rates of pyrophosphate exchange of crude extracts of *A. californica* and *A. hippocastanum* were proportional to the protein concentration of the extracts up to 0.4 and 0.9mg of protein/ml respectively. Synthetase activity in crude extracts was always assayed below these limits because pyrophosphate exchange decreases if the concentration of crude extract exceeds a critical value (Anderson & Rowan, 1966; Anderson & Fowden, 1969). The specific activities of purified phenylalanyl-tRNA synthetases were independent of enzyme concentration.

Determination of protein. Protein in crude extracts and $(NH_4)_2SO_4$ fractions prepared from crude extracts was precipitated with 10% (w/v) trichloroacetic acid, washed twice with acetone, solubilized in 0.1M-NaOH and the protein determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Protein solubilized with KCl from protamine sulphate precipitates of *A. hippocastanum* and *A. glabra* also was precipitated with 10% (w/v) trichloroacetic acid and washed twice with acetone. However, the precipitate would dissolve only in 2M-NaOH and, as this concentration exceeds the maximum concentration of NaOH permissible in the method of Lowry *et al.* (1951), the method of Ellman (1962) was then used. The method of Warburg & Christian (1941) was used to determine protein in all other fractions. Crystalline bovine serum albumin was used as a standard for protein determination.

Determination of K_m values. Reaction rates, v, were measured at a minimum number of ten concentrations of substrate S. K_m was calculated by the method of Lineweaver & Burk (1934) on an Olivetti desk computer by the least-squares method.

RESULTS

Purification of phenylalanyl-tRNA synthetases of A. californica, A. hippocastanum, A. glabra *and* A. parviflora. The average results of the purification of these enzymes are shown in Table 3. The simple two-stage purification of the phenylalanyl-tRNA synthetase of *A. californica* effected a 30-fold increase in specific activity and decreased the endogenous pyrophosphate exchange activity to a very low value. This treatment also decreased the activity of all the other synthetases, though the effectiveness of this treatment for removing contaminating synthetases was better for seeds harvested in 1968 than in 1967 (Table 2). When seeds harvested in 1967 were used, fractions prepared at concentrations of acetone greater than 35% contained little phenylalanyl-tRNA synthetase but showed high pyrophosphate exchange activity when an amino acid mixture was used as substrate (Table 1). The main synthetase in these fractions was valyl-tRNA synthetase (Anderson & Fowden, 1970). The activity of each synthetase during purification of phenylalanyl-tRNA synthetase from seeds of *A. californica* (1967 harvest) is shown in Table 2; in this experiment the activity of the valyl-tRNA synthetase is abnormally high because protein was collected by precipitation with 25–35% acetone (cf. routine preparations, 25–32% acetone for 1967 seeds). However, contaminating valyl-tRNA synthetase present in precipitates obtained after acetone fractionation could be separated easily on DEAE-cellulose (Table 2). The sum of the pyrophosphate exchange activities measured for individual synthetases (measured separately but excluding the phenylalanyl-tRNA synthetase) invariably exceeded that measured when the synthetases were measured collectively with an amino acid mixture as substrate (Table 2); this situation is commonly encountered when these synthetases are assayed.

The method used to purify the phenylalanyl-tRNA synthetase of *A. californica* was not suitable for purifying the enzyme from *A. hippocastanum.* Phenylalanyl-tRNA synthetase activity was precipitated in all ammonium sulphate fractions prepared from crude extracts of *A. hippocastanum*; the specific activity of all fractions was similar to that of the crude extract. Further, the specific

Table 3. *Average values for the purification of the phenylalanyl-tRNA synthetases from cotyledons of seeds of* A. californica, A. hippocastanum, A. glabra *and* A. parviflora

Species	Treatment	Units of phenylalanyl-tRNA synthetase recovered in crude extracts/g fresh wt.	Recovery of phenylalanyl-tRNA synthetase (% of enzyme activity in crude extract)	Endogenous pyrophosphate exchange activity (% of that of phenylalanyl-tRNA synthetase in each fraction)	Contaminating aminoacyl-tRNA synthetases (% of phenylalanyl-tRNA synthetase in each fraction)	Sp. activity (nmol/min per mg of protein)
A. californica (1967 harvest)	Crude extract	33–45	100	570	830	3.0
	$(NH_4)_2SO_4$(0.293–0.342 g/ml)		67	60	320	16
	Acetone (25–32%)		15	3	15	90
A. hippocastanum	Crude extract	11–18	100	450	1550	1.2
	Protamine sulphate and Sephadex G-50		20	20	170	10
	$(NH_4)_2SO_4$(0–60% satn.)		15	15	150	15
	DEAE-cellulose (phenylalanyl-tRNA synthetase peak)		5	0	35	85
A. glabra	Crude extract	22–30	100	165	740	1.5
	Protamine sulphate and Sephadex G-50		28	8	160	25
	$(NH_4)_2SO_4$(0–60% satn.)		20	2	165	37
A. parviflora	Crude extract	18–22	100	300	620	2.0
	$(NH_4)_2SO_4$(0.293–0.342 g/ml)		70	35	300	25
	Acetone (25–40%)		25	1	35	220

activity of the phenylalanyl-tRNA synthetase of *A. hippocastanum* was destroyed when precipitated with acetone even when all the ammonium sulphate was removed. Preliminary experiments with protamine sulphate to precipitate RNA revealed that much of the phenylalanyl-tRNA synthetase of *A. hippocastanum* was also precipitated and the activity could be solubilized with 1 M-potassium chloride or 1 M-lithium chloride. When potassium chloride extracts were passed through Sephadex G-50 to remove potassium chloride and the yellow pigment, 90% of the phenylalanyl-tRNA synthetase activity extracted from the protamine sulphate precipitate with potassium chloride was recovered, but there was a large rise in the activity of the other aminoacyl-tRNA synthetases and the endogenous activity also increased. Addition of potassium chloride to fractions eluted from Sephadex decreased both the endogenous activity and the amino acid-dependent activity of contaminating aminoacyl-tRNA synthetases (Fig. 1). This effect was also observed in preparations of *A. glabra*. For this reason 0.5 M-potassium chloride was always included in reaction mixtures when the phenylalanyl-tRNA synthetases of *A. hippocastanum* and *A. glabra* were assayed.

Fig. 1. Effect of KCl concentration present in the incubation medium on activity of phenylalanyl-tRNA synthetase (▲), other aminoacyl-tRNA synthetases (●) and endogenous pyrophosphate exchange (■) of a partially purified preparation from *A. hippocastanum*. The preparation used in this experiment was not previously chromatographed on DEAE-cellulose to separate the phenylalanyl-tRNA synthetase from contaminating aminoacyl-tRNA synthetases.

Table 4. *Activity of the individual aminoacyl-tRNA synthetases during purification of the phenylalanyl-tRNA synthetase of* A. hippocastanum

Results are expressed as percentages of the phenylalanine-dependent pyrophosphate exchange activity at each stage of purification

	Crude extract	$(NH_4)_2SO_4$ (0–60% satn.) treatment after protamine sulphate and Sephadex G-50		DEAE-cellulose treatment (phenylalanyl-tRNA synthetase peak)	
		−KCl	+KCl	−KCl	+KCl
Alanine	15	3	0	5	0
Arginine	26	6	0	0	0
Asparagine	3	13	0	0	0
Aspartic acid	60	1	0	0	0
Cysteine	102	1	0	4	1
Glutamic acid	70	6	0	7	0
Glutamine	18	0	0	0	0
Glycine	19	2	0	0	2
Histidine	15	23	3	0	2
Isoleucine	793	421	81	168	13
Leucine	972	548	128	200	24
Lysine	97	0	0	0	0
Methionine	36	0	0	0	0
Phenylalanine	100	100	100	100	100
Proline	95	0	0	0	0
Serine	61	0	12	22	27
Threonine	274	110	93	40	34
Tryptophan	107	24	11	6	8
Tyrosine	82	0	0	0	0
Valine	506	112	13	31	7
Complete mixture less phenylalanine		614	273	316	60

Leucyl-tRNA synthetase, present as a major contaminant at this stage of purification, was inhibited strongly by potassium chloride (Table 4).

Chromatography on DEAE-cellulose partially separated phenylalanyl-tRNA synthetase from two contaminating aminoacyl-tRNA synthetases (Fig. 2), and by careful selection fractions with minor contaminants were obtained (Table 4). These main contaminating enzymes were threonyl-tRNA synthetase (peak I) and leucyl-tRNA synthetase (peak II) (Anderson & Fowden, 1970). The activity of the aminoacyl-tRNA synthetases at each stage of purification and the effect of potassium chloride on the activity of these synthetases are summarized in Table 4. The increase in specific activity of the phenylalanyl-tRNA synthetase from *A. hippocastanum* varied with the harvest; the enzyme was purified up to 250-fold from seeds harvested in 1968 and up to 800-fold (specific activity 1260 units/mg of protein) from seeds harvested in 1969. As with *A. californica*, the sum of the pyrophosphate exchange activities measured for the individual aminoacyl-tRNA synthetases of *A. hippocastanum* exceeded the exchange occurring when synthetases were assayed collectively with an amino acid mixture.

The leucyl-tRNA synthetase activity in purified preparations of phenylalanyl-tRNA synthetase from *A. parviflora* did not exceed 0.5% of the pyrophosphate exchange activity of the phenylalanyl-tRNA synthetase and consequently potassium chloride was omitted from assays.

No phenylalanyl-tRNA synthetase activity was obtained when ATP was omitted from assays of purified phenylalanyl-tRNA synthetases of all species.

Properties of the phenylalanyl-tRNA synthetases. The K_m values for ATP and phenylalanine for the enzymes from all species are listed in Table 5.

The rate of pyrophosphate exchange effected by the phenylalanyl-tRNA synthetase from *A. californica* at 30°C and 35°C was constant for 16min. The rate at 35°C was approx. 13% greater than at 30°C. The rate of pyrophosphate exchange at 40°C was initially greater than at 35°C but the rate decreased after 4min and pyrophosphate exchange ceased after 16min. Consequently standard assays were conducted at 35°C for 15min. Pyrophosphate exchange increased with concentration of pyrophosphate (of constant specific radioactivity) up to 2mM but decreased at higher concentrations; the activity with 4mM-pyrophosphate was only 50% of that

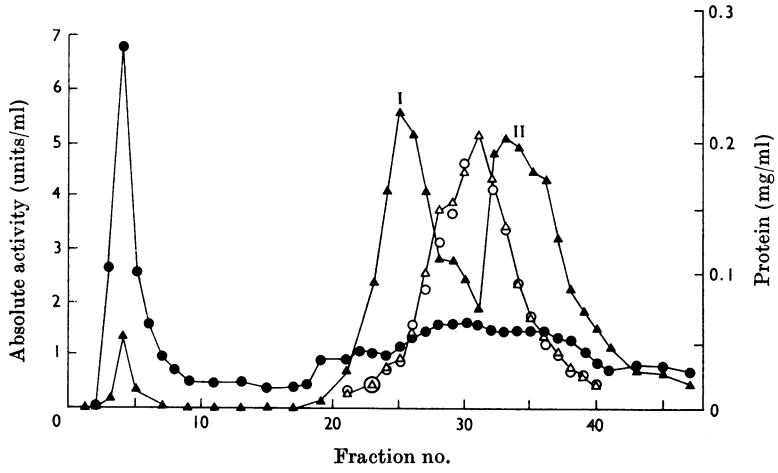


Fig. 2. Elution pattern of protein (●), phenylalanyl-tRNA synthetase (△) and other aminoacyl-tRNA synthetases of *A. hippocastanum* when chromatographed on DEAE-cellulose. Pyrophosphate exchange activity was measured with, as substrate, phenylalanine (△), 20mM-AMHA (○) and an amino acid mixture from which phenylalanine was omitted (▲). Peak I was predominantly threonyl-tRNA synthetase and peak II was predominantly leucyl-tRNA synthetase.

Table 5. *Summary of the properties of the phenylalanyl-tRNA synthetases from five species of* Aesculus

Values for K_m were computed from ten values of [S] and v in single experiments and are expressed ± S.E.M.

Species	K_m (ATP) (mM)	K_m (phenylalanine) (mM)	K_m (AMHA) (mM)	K_m (AMHA)/ K_m (phenylalanine) ratio	V (AMHA)/ V (phenylalanine) ratio
A. californica	0.33 ± 0.07	0.105 ± 0.008	3.2 ± 0.4	30	0.3
A. glabra	0.36 ± 0.02	0.049 ± 0.002	1.8 ± 0.2	36	0.9
A. hippocastanum	0.28 ± 0.03	0.031 ± 0.001	1.18 ± 0.13	38	1.0
A. parryi	0.38 ± 0.04	0.019 ± 0.001	0.89 ± 0.07	47	1.0
A. parviflora	0.28 ± 0.01	0.016 ± 0.001	0.68 ± 0.05	43	1.0

obtained with 2mM-pyrophosphate. Pyrophosphate exchange also increased with ATP concentration up to 2mM; higher concentrations of ATP (up to 6mM) were not inhibitory. When equimolar concentrations of ATP and pyrophosphate were used over the range 1–6mM, pyrophosphate exchange was greatest when 2mM-ATP and 2mM-pyrophosphate were used. When tris–maleic acid–potassium hydroxide buffer (Colowick & Kaplan, 1955) was used, maximum pyrophosphate exchange was observed at approx. pH 7.0. Phenylalanyl-tRNA synthetase activity could not be detected at pH 5.3, but loss of activity at alkaline pH was less marked; approx. 40% of the maximum activity observed at pH 7.0 could be detected at pH 9.0.

The rate of pyrophosphate exchange of the phenylalanyl-tRNA synthetase from *A. hippocastanum* at 30°C and 35°C was constant for 20min. The rate at 35°C was 13% greater than at 30°C. The rate of exchange at 40°C was higher than at 35°C but decreased after 12min. Hence a standard assay of 15min at 35°C was selected. The phenylalanyl-tRNA synthetase of *A. hippocastanum* resembles the enzyme from *A. californica* in many respects. Pyrophosphate exchange increased with concentration of pyrophosphate up to 2mM but decreased at higher concentrations. Pyrophosphate exchange increased with concentration of ATP up to 2mM and remained constant at concentrations of ATP up to 4mM. When tris–maleic acid–potassium hydroxide buffer (Colowick & Kaplan, 1955) was used, maximum pyrophosphate exchange occurred at approx. pH 7.0. No activity was obtained at pH 5.3, but 52% of the maximum activity was observed at pH 9.0.

Activation of AMHA by the phenylalanyl-tRNA synthetases from representative Aesculus *species.* AMHA served as a substrate for all the phenylalanyl-tRNA synthetases tested (Table 5). V varied with the species, being highest for *A.*

hippocastanum, *A. parryi* and *A. parviflora* (100% of the phenylalanine value) and least for *A. californica* (30% of the phenylalanine value). The K_m for AMHA also varied with the species, *A. parviflora* showing the highest affinity and *A. californica* the least. In all species the affinity for AMHA was 30–47-fold less than the affinity for phenylalanine. When saturating concentrations of AMHA were used with the phenylalanyl-tRNA synthetase of *A. californica* in the presence of a barely saturating concentration of phenylalanine, pyrophosphate exchange activity was less than that observed when only phenylalanine was used as substrate. This suggests that the two substrates are competing for the one active site on the phenylalanyl-tRNA synthetase enzyme. The same effect was observed with the enzymes from *A. hippocastanum*, *A. glabra*, *A. parryi* and *A. parviflora*, but with these species a non-saturating concentration of AMHA was used because V for AMHA relative to phenylalanine is so high that substrate competition is not observed unless the concentration is decreased.

Further support for the idea that AMHA is a true substrate for the phenylalanyl-tRNA synthetases of the various *Aesculus* species comes from several lines of evidence. Activation of AMHA relative to phenylalanine was constant during all stages of purification except for crude extracts. Pyrophosphate exchange activity of crude extracts of *A. californica* and *A. hippocastanum* with AMHA as substrate was often less than the endogenous activity, but this could be due to competition between AMHA and endogenous substrates such as phenylalanine and acetate and sulphate, which participate in various pyrophosphate exchange reactions.

During elution from DEAE-cellulose columns, AMHA-dependent pyrophosphate exchange was invariably effected only by those fractions containing phenylalanyl-tRNA synthetase. This is best illustrated by an experiment carried out with the phenylalanyl-tRNA synthetase of *A. hippocastanum* (Fig. 2); the elution profile also shows that V for AMHA-dependent activity approximates V for phenylalanine-dependent activity in this species. Similar observations were made for extracts from *A. californica*, but in this case the activation of AMHA was approx. 30% of the phenylalanine value (Fig. 3). The phenylalanine- and AMHA-dependent activities were separated completely from a major contaminant (valyl-tRNA synthetase), which was not retarded on DEAE-cellulose, but other minor contaminating synthetases were still present. Nevertheless, the activation of AMHA cannot be due to such contaminating aminoacyl-tRNA synthetases because a preparation of phenylalanyl-tRNA synthetase from *A. californica* (1968 harvest), containing contaminating synthetases in only trace amounts, still utilized AMHA at 34% of the rate determined for phenylalanine; pyrophosphate exchange activity due to the most active contaminating synthetase (leucyl-tRNA

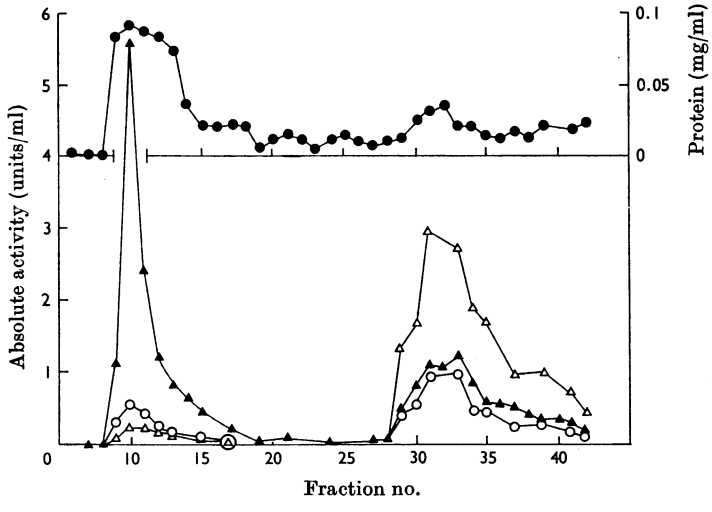


Fig. 3. Elution pattern of protein (●), phenylalanyl-tRNA synthetase (△) and other aminoacyl-tRNA synthetases of *A. californica* when chromatographed on DEAE-cellulose. Pyrophosphate exchange activity was measured with, as substrate, phenylalanine (△), 10mM-AMHA (○) and an amino acid mixture from which phenylalanine was omitted (▲).

synthetase) was only 5% of the phenylalanine-dependent pyrophosphate exchange (Table 2).

The activation of AMHA relative to phenylalanine was constant for phenylalanyl-tRNA synthetases of *A. californica* and *A. hippocastanum*, irrespective of pH.

Effect of ions on the phenylalanyl-tRNA synthetases of Aesculus *species.* The enzyme from *A. hippocastanum* was insensitive to potassium chloride in the assay mixture up to a concentration of 1 M (Fig. 1), though sometimes a small stimulation (up to 30%) was observed with 0.5 M-potassium chloride. Enzyme activity was also increased slightly (up to 20%) by ammonium chloride (0.67 M), potassium bromide (0.67 M) and potassium nitrate (0.67 M). Enzyme activity was insensitive to sodium chloride (0.67 M), but 0.33 M-magnesium chloride decreased the activity by 15%. Calcium chloride (0.33 M) completely inhibited the enzyme activity, presumably by competing with the low concentration of Mg^{2+} required as a cofactor for maximum enzyme activity (Peterson, 1967). The phenylalanyl-tRNA synthetases from *A. glabra*, *A. parviflora* and *A. parryi* were also insensitive to 0.5 M-potassium chloride, though sometimes a small stimulation (up to 20%) was observed. By contrast the activity of the phenylalanyl-tRNA synthetase from *A. californica* was decreased by 50% in the presence of 0.5 M-potassium chloride.

Activation of other compounds by the phenylalanyl-tRNA synthetases of A. hippocastanum *and* A. californica. The phenylalanyl-tRNA synthetase of *A. hippocastanum* was usually not fully separated from several contaminating synthetases (Table 4). Consequently substrate-dependent pyrophosphate exchange was not sufficient in itself to imply that a compound was activated by the phenylalanyl-tRNA synthetase, and evidence indicating competition with phenylalanine at the active site of the enzyme was considered essential. Compounds fulfilling these criteria are listed in Table 6.

Three compounds are listed in Table 6 as suspected analogues. These compounds, when used as the sole substrate, induced pyrophosphate exchange, but did not significantly diminish pyrophosphate exchange when added to normal assay systems containing phenylalanine. However, when the suspected analogues were added with phenylalanine, the pyrophosphate exchange did not exceed the pyrophosphate exchange obtained with phenylalanine alone. Further, the pyrophosphate exchange measured with methallylglycine [(B)(iv) in Table 6] and β-pyrid-2-ylalanine [(D)(ii) in Table 6] was higher than that conceivably attributable to any of the contaminating synthetases (Table 4) except threonyl-tRNA synthetase, which showed additive pyrophosphate exchange with these substrates. Therefore it is improbable that activation of the suspected analogues was effected by contaminating aminoacyl-tRNA synthetases. One reason for the lack of competition could be that these analogue substrates were contaminated with phenylalanine, but this is extremely unlikely since the compounds were chemically synthesized by methods that could not yield phenylalanine. Certainly phenylalanine was absent from these compounds when examined by paper chromatography.

Although most purified preparations of phenylalanyl-tRNA synthetase prepared from *A. hippocastanum* contained leucyl-tRNA synthetase and valyl-tRNA synthetase, one preparation made from seed stored for 9 months at 2°C contained no contaminating synthetases. This preparation (purified 200-fold) activated crotylglycine [(B)(iii) in Table 6] and 2-aminohex-4,5-dienoic acid [(B)(ii) in Table 6].

Compounds listed in Table 6 as not inducing pyrophosphate exchange also did not decrease pyrophosphate exchange when phenylalanine was used as substrate: other compounds such as uracil-5-ylalanine, 1-aminocyclopentane-1-carboxylic acid, and 1-amino-2-nitrocyclopentane-1-carboxylic acid showed similar behaviour.

There is less ambiguity about the activation of alternative substrates by the phenylalanyl-tRNA synthetase of *A. californica*, because this enzyme could be obtained relatively free from contaminating synthetases (Table 2). All the compounds, activated by the phenylalanyl-tRNA synthetase from *A. hippocastanum*, which were also tested with the enzyme from *A. californica*, induced pyrophosphate exchange though in all cases the rate of activation for specific concentrations of substrate was less for the enzyme from *A. californica* than the enzyme from *A. hippocastanum* (Table 6). 6-Hydroxy-AMHA and β-uracil-3-ylalanine (willardine) were not activated by the phenylalanyl-tRNA synthetase of *A. californica*.

The phenylalanyl-tRNA synthetase from *A. parviflora* activated 2-amino-5-methylhex-4-enoic acid [(B)(vi) in Table 6]. The pyrophosphate exchange at a concentration of 10 mM was 69% of the phenylalanine-dependent exchange.

DISCUSSION

Purification of specific aminoacyl-tRNA synthetases from plant tissues entails: (1) removal of endogenous substrates; it is commonplace to find no amino acid-dependent exchange in crude extracts of plant tissues; (2) removal of unwanted (contaminating) aminoacyl-tRNA synthetases; (3) removal of inactive protein to increase specific activity. The increase in specific activity of the phenylalanyl-tRNA synthetases of *Aesculus* species

reported in this paper is not very high even for the most highly purified enzyme from *A. hippocastanum* (up to 840-fold), but nevertheless the increase in specific activity of the phenylalanyl-tRNA synthetases of *A. californica*, *A. parviflora* and *A. hippocastanum* are comparable with the most highly purified aminoacyl-tRNA synthetases prepared from plant tissues (cf. Peterson & Fowden, 1965; Legocki & Pawelkiewicz, 1967; Smith & Fowden, 1968).

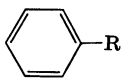
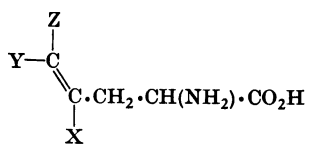
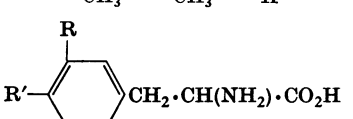
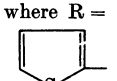
The more important aspect of purification was the removal of contaminating aminoacyl-tRNA synthetases; this is essential when working with analogue substrates, since V for the activation of an analogue is usually appreciably less than V determined for the normal substrate of a particular synthetase. The phenylalanyl-tRNA synthetase prepared from *A. californica* by fractionation with ammonium sulphate and acetone was virtually free of contaminating aminoacyl-tRNA synthetases (Table 2). Attempts to remove contaminating synthetases from the phenylalanyl-tRNA synthetase of *A. hippocastanum* by simple procedures were less successful owing to persistence of threonyl-tRNA synthetase and leucyl-tRNA synthetase through the later stages of purification. These enzymes were partially separated on DEAE-cellulose, but leucyl-tRNA synthetase activity in the fractions rich in phenylalanyl-tRNA synthetase remained high (up to twice as active as phenylalanyl-tRNA synthetase in some experiments; e.g. see Table 4). However, the activity of the leucyl-tRNA synthetase was decreased to a low value when 0.5M-potassium chloride was included in the assay medium (Table 4). The results in Table 4 indicate that high isoleucine-dependent pyrophosphate exchange activity also persisted into the later stages of purification of the phenylalanyl-tRNA synthetase of *A. hippocastanum*, and was inhibited by 0.5M-potassium chloride in the same way as was the leucyl-tRNA synthetase. However, the pyrophosphate exchange activity measured with isoleucine as substrate was probably due to activation of isoleucine by the leucyl-tRNA synthetase, though the affinity of this enzyme for isoleucine is 100-fold less than for leucine (Anderson & Fowden, 1970).

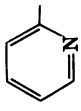
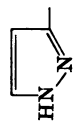
The phenylalanyl-tRNA synthetase from all species examined had approximately the same affinity for ATP (Table 5), and the synthetases from *A. californica* and *A. hippocastanum* had similar pH optima and temperature-lability. The affinity of the phenylalanyl-tRNA synthetases for AMHA was 30–47-fold less than for phenylalanine in all species, suggesting that the configuration at the active centre of the enzyme was closely similar in all species. Smith & Fowden (1968) reported that the affinity of phenylalanyl-tRNA synthetase of *Phaseolus aureus* for AMHA is also 40-fold less than for phenylalanine.

The phenylalanyl-tRNA synthetase from *A. californica* has a lower affinity for phenylalanine than have the synthetases from the other species, and consequently a lower affinity for AMHA. The synthetase from *A. californica* also has a lower V (AMHA)/V(phenylalanine) ratio than have the synthetases from the other species (Table 5). Consequently the activation of AMHA, at specified concentrations, by the phenylalanyl-tRNA synthetase of *A. californica* is less than that occurring with enzyme prepared from species of *Aesculus* that do not produce AMHA (Fig. 4), or with *P. aureus* enzyme (Smith & Fowden, 1968). These results suggest that the phenylalanyl-tRNA synthetase of *A. californica* is unique in activating AMHA to a smaller extent than do the synthetases from other species, and it could represent a special adaptation serving to limit the incorporation of AMHA into protein by discrimination at this first stage of protein synthesis. If it is assumed that AMHA is not spatially separated from the phenylalanyl-tRNA synthetase in *A. californica* (the subcellular distributions of both AMHA and phenylalanyl-tRNA synthetase are unknown), then clearly a second and more specific selection must operate at a later stage to prevent the incorporation of AMHA into protein.

The mechanism for excluding AMHA from protein of *A. californica* then differs from the mechanism for excluding azetidine-2-carboxylic acid (in lieu of proline) from the protein of various lileaceous plants that produce this toxic imino acid; the prolyl-tRNA synthetase of these species fails to activate azetidine-2-carboxylic acid, though this imino acid is activated by the prolyl-tRNA synthetase of *P. aureus*, a species that does not produce azetidine-2-carboxylic acid and that is sensitive to exogenously supplied azetidine-2-carboxylic acid (Peterson & Fowden, 1965). Mimosine, which is found in members of the genera *Leucaena* and *Mimosa*, is activated by the phenylalanyl-tRNA synthetases in these species, but mimosine is excluded from protein by a different mechanism. The phenylalanyl-tRNA synthetase of *Leucaena leucocephala* exhibits a V (mimosine/V (phenylalanine) ratio greater than that obtained with the phenylalanyl-tRNA synthetase of *P. aureus*, a species that does not produce mimosine (Smith & Fowden, 1968). This situation should favour the incorporation of mimosine into the protein of species producing mimosine. However, transfer of [^{14}C]phenylalanine to tRNA by the phenylalanyl-tRNA synthetase of *L. leucocephala* was not impaired by the presence of high concentrations of mimosine, and Smith & Fowden (1968) concluded that mimosine was excluded from protein during

Table 6. *Relationship between structure and activity of compounds used as substrates for the phenylalanyl-tRNA synthetases of* A. hippocastanum *and* A. californica

		Compound	v [% of V (phenylalanine)]	
			A. hippocastanum	*A. californica*
(A)	 where R =			
(i)	$CH_2 \cdot CO \cdot CO_2H$	Phenylpyruvic acid (10mM)	0	
(ii)	$CH_2 \cdot CH(NH_2) \cdot CO_2H$	D-Phenylalanine (10mM)	0	
(iii)	$CH(OH) \cdot CH(NH_2) \cdot CO_2H$	DL-β-Phenylserine (10mM)	51*	
(iv)	$CH(NH_2) \cdot CO_2H$	L-Phenylglycine (2.5mM)	0	
(v)	$CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$	DL-2-Amino-4-phenylbutyric acid (5mM)	0	
(vi)	$CH(OH) \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$	DL-2-Amino-4-hydroxy-4-phenylbutyric acid (5mM)	0	
(B)				
	where X = Y = Z =			
(i)	H H H	DL-Allylglycine (10mM)	0	
(ii)	H CH_2	L-2-Aminohex-4,5-dienoic acid (5mM)	23	
(iii)	H CH_3 H	DL-Crotylglycine (5mM)	29	15
(iv)	CH_3 H H	DL-Methallylglycine (10mM)	35†	8
(v)	C_2H_5 H H	DL-Ethallylglycine (10mM)	40*	22
(vi)	H CH_3 CH_3	L-2-Amino-5-methylhex-4-enoic acid (10mM)	48*	
(viii)	CH_3 CH_3 H	DL-AMHA (20mM)	100*	32
(C)				
	where R = R′ =			
(i)	F H	DL-*m*-Fluorophenylalanine (5mM)	52*	
(ii)	CO_2H H	L-*m*-Carboxyphenylalanine (5mM)	0	
(iii)	H $O \cdot CH_3$	DL-*p*-Methoxyphenylalanine (5mM)	42*	17
(iv)	H OH	L-Tyrosine (5mM)	0	
(v)	CN H	DL-*m*-Cyanophenylalanine (10mM)	0	
(D)	$R \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$ where R =			
(i)		DL-β-Thien-2-ylalanine (5mM)	68*	64

(ii)		DL-β-Pyrid-2-ylalanine (10mM)	37
(iii)		DL-β-Pyrazol-1-ylalanine (10mM)	50†
(iv)		DL-β-Pyrazol-3-ylalanine (10mM)	10†
(v)		DL-β-1,2,4-Thiazol-3-ylalanine (10mM)	0
			0

* Competition between these substrates and phenylalanine was demonstrated by using phenylalanyl-tRNA synthetase from *A. hippocastanum*. Conditions used to demonstrate competition were: 10mM-phenylserine with 2mM-phenylalanine, 10mM-ethallylglycine with 1mM-phenylalanine, 10mM-2-amino-5-methylhex-4-enoic acid with 2mM-phenylalanine, 10mM-AMHA with 1mM-phenylalanine, 5mM-fluorophenylalanine with 2mM-phenylalanine, 5mM-methoxyphenylalanine with 0.5mM-phenylalanine and 5mM-thienylalanine with 0.5mM-phenylalanine.

† Suspected analogues: competition between these substrates and phenylalanine could not be demonstrated.

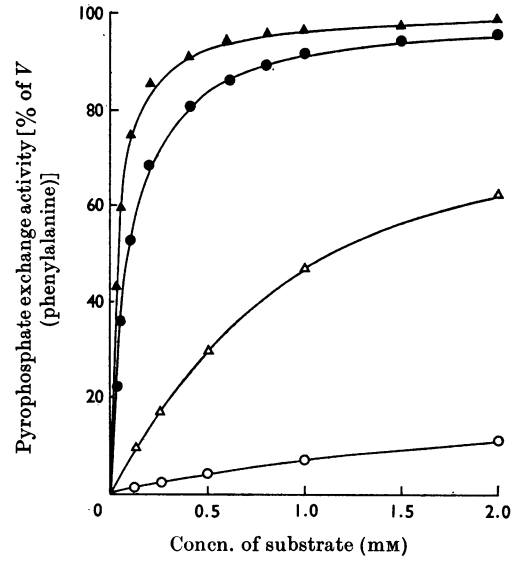


Fig. 4. Relation between substrate concentration and pyrophosphate exchange of the phenylalanyl-tRNA synthetases of *A. hippocastanum* and *A. californica* for the substrates phenylalanine and AMHA. The results were calculated from the values of K_m and V cited in Table 5. Pyrophosphate exchange activity is expressed as a percentage of the theoretical maximum exchange obtainable with phenylalanine as substrate. *A. hippocastanum* enzyme: ▲, with phenylalanine; △, with AMHA; *A. californica* enzyme: ●, with phenylalanine; ○, with AMHA.

the second stage of the activation reaction, i.e. the transfer of the aminoacyl residue from the adenylate to $tRNA^{Phe}$. This could be the stage at which discrimination against AMHA occurs, so excluding it from the protein of *A. californica*.

Other differences in the properties of phenylalanyl-tRNA synthetases isolated from the various *Aesculus* species were evident. For instance, the enzyme from *A. californica* was inhibited by 0.5M-potassium chloride whereas the enzymes from the other species were either insensitive or slightly stimulated by K^+. With the exception of thienylalanine, the enzyme from *A. californica* activates other phenylalanine analogues, relative to the activation of phenylalanine, at about half the rate of the enzyme from *A. hippocastanum* (Table 6). A similar comparison of the activities of the enzymes from *A. hippocastanum* and *A. parviflora* towards one analogue substrate, namely 10mM-2-amino-5-methylhex-4-enoic acid, indicated its utilization at rates equivalent to 48 and 69% respectively of V for phenylalanine. Evidence of this type suggests that those phenylalanyl-tRNA synthetases

exhibiting the highest affinity for phenylalanine show the lowest specificity towards analogue compounds. If this contention were generally true, a gradation in specificity would exist, the phenylalanyl-tRNA synthetase from *A. californica* having the highest specificity towards amino acid substrates and the enzyme from *A. parviflora* the least.

Table 6 summarizes the results of activation of compounds structurally related to phenylalanine by the phenylalanyl-tRNA synthetase of *A. hippocastanum*. An L-alanyl or L-seryl side chain is essential for activity; lengthening or shortening the chain abolishes activity (Table 6A). These results can be interpreted to mean that a double bond is essential between C-4 and C-5.

Allylglycine [(B)(i) in Table 6] was not activated by phenylalanyl-tRNA synthetase of *A. hippocastanum*. Addition to allylglycine of one or two carbon atoms attached by single or double bonds, either at C-4 or in the *trans* position relative to the alanyl side chain at C-5 (e.g. crotylglycine, ethallylglycine, methallylglycine and 2-aminohex-4,5-dienoic acid), conferred activity equivalent to 25–40% of that measured with saturating concentrations of phenylalanine (Table 6B). Addition of carbons to allylglycine at both C-4 and the *trans* C-5 positions (AMHA) enhanced the activity to 100% of that for phenylalanine. Saturation of the double bond in AMHA gave rise to an inactive compound, 2-amino-4-methylhexanoic acid. The minimum structural features necessary in a non-cyclic phenylalanine analogue therefore include an unbranched chain of five carbon atoms, a double bond between C-4 and C-5 and a sixth carbon atom attached either at C-4 or in a *trans* configuration at C-5. These results also imply that there are substrate receptor sites on the phenylalanyl-tRNA synthetase from *A. hippocastanum* that recognize (1) the branched carbon atom attached to C-4 and (2) the terminal carbon atom(s) attached in a *trans* position at C-5. When one site is occupied activation occurs at 25–40% of the rate measured for phenylalanine; when both sites are occupied activation occurs at 100% the rate of phenylalanine. When methyl groups are attached to both the *cis* and *trans* positions at C-5 but not at C-4 (i.e., as in 2-amino-5-methylhex-4-enoic acid), an intermediate extent of compound activation was observed. The possession of an extra hydrophobic group on C-5 (cf. crotylglycine) seemingly enhanced substrate affinity, possibly by permitting additional non-polar binding at the active centre.

Compounds with small non-polar substituent groups on the phenyl ring of phenylalanine, e.g. *m*-fluorophenylalanine, *p*-methoxyphenylalanine, acted as substrates of the phenylalanyl-tRNA synthetase of *A. hippocastanum*, but substitution by polar groups led to inactive compounds (Table 6C).

A number of other β-substituted alanines, in which a heterocyclic ring system replaced the phenyl group of phenylalanine, also served as substrates for this enzyme: such compounds included β-thien-2-ylalanine [(D)(i) in Table 6], β-pyrid-2-ylalanine [(D)(ii) in Table 6] and β-pyrazol-1-ylalanine [(D)(iii) in Table 6]. However, if the ring system replacing the phenyl group contained a free basic nitrogen atom, as in triazole or pyrazole derivatives linked through the C-3 atom, then the resulting β-substituted alanine was not active as a substrate.

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