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STUDIES ON THE SPECIFICITIES OF THE PHENYLALANYL- AND TYROSYL-sRNA SYNTHETASES FROM PLANTS

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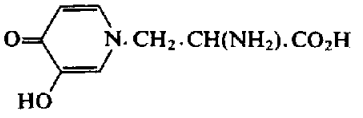
Abstract—The phenylalanyl- and tyrosyl-sRNA synthetases of mung bean seed have been partially purified and their ability to utilize a range of amino acid analogues as substrates determined. The substrate specificity of the phenylalanyl-enzyme seems to be less exacting than that of the tyrosyl-enzyme, a finding in agreement with previous observations using animal and microbial enzymes. Mimosine, a toxic amino acid present in *Mimosa* and *Leucaena* species, served as a substrate for the phenylalanyl-, but not the tyrosyl-sRNA synthetase from mung bean. However, the transfer of ^{14}C-phenylalanine to tRNA catalysed by its synthetase enzyme was not impaired by the presence of high concentrations of mimosine; *Leucaena* phenylalanyl-sRNA synthetase also activated mimosine, but it is concluded that mimosine-induced toxicities do not arise primarily by interference with phenylalanine incorporation into protein molecules. 2-Amino-4-methylhex-4-enoic acid, a natural product of *Aesculus californica*, behaved as a phenylalanine analogue, being activated by the phenylalanyl-sRNA synthetase of mung bean at rates comparable to that determined for phenylalanine itself.

INTRODUCTION

THIS investigation developed from earlier studies concerned with the biochemical mechanisms underlying the toxicity of mimosine to animals and plants.



(I)

Mimosine (I) is present in high amounts in the seeds of species of two related genera, *Mimosa* and *Leucaena*.[1] Lower concentrations of the amino acid occur in the vegetative parts of these plants, and toxic symptoms have been described frequently in animals grazing upon the foliage and seed pods, especially from *L. leucocephala*.[2] The symptoms include growth inhibitions, hair loss and infertility. Similarly mimosine may completely inhibit the growth of cultures of *Escherichia coli*[3] and mung bean seedlings.[4]

A discussion of the ways in which mimosine may produce growth inhibition and other toxic symptoms in animals by antagonizing intermediary reactions dependent upon ferrous ions or pyridoxal phosphate or, additionally, in plants by acting as a precursor of the equally toxic 3,4-dihydroxypyridine, has been given earlier.[4] However, the observation that mimosine-induced growth inhibition could be reduced or completely eliminated by supplying

[1] J. L. BREWBAKER and J. W. HYLIN, *Crop Sci.* **5**, 348 (1965).
[2] M. P. HEGARTY, P. G. SCHINCKEL and R. D. COURT, *Australian J. Agri. Res.* **15**, 153 (1964).
[3] S. SUDA, *Bot. Mag., Tokyo* **73**, 142 (1960).
[4] I. K. SMITH and L. FOWDEN, *J. Exp. Botany* **17**, 750 (1966).

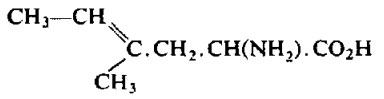
animals with additional phenylalanine and tyrosine[5,6] suggested that the antagonistic action of mimosine might be upon processes utilizing phenylalanine and/or tyrosine, including the early steps of protein synthesis involving the phenylalanyl- and tyrosyl-sRNA synthetase enzymes.

Mung bean seeds, whose growth is inhibited strongly by mimosine, form a good source of aminoacyl-sRNA synthetases.[7] The seeds have been used in the present investigation for the separation of the phenylalanyl- and tyrosyl-enzymes to test whether mimosine acts as a substrate for, or inhibitor of, either of these synthetases. In addition, the amino acid substrate specificity of each enzyme has been surveyed using a range of structurally-related compounds, and some comparisons have been made with the corresponding phenylalanyl-sRNA synthetase isolated from *Leucaena* seeds.

Previous work with bacterial and animal systems has indicated that the phenylalanyl-sRNA synthetase is less stringent in selecting its amino acid substrate than is the corresponding tyrosyl-enzyme. Edelson *et al.*[8] have summarized the degrees of structural variation permissible in compounds antagonizing phenylalanine utilization by *E. coli*. Most, but not all, of the substances exhibiting such antimetabolite properties were accepted as substrates by the phenylalanyl-sRNA synthetase of this organism. Normally a substrate must contain an alanyl side-chain whose β-carbon atom is joined to and coplanar with a ring system structurally similar to the phenyl group. In practice, pyridinyl, thienyl, thiazolyl, cyclopentenyl and cyclohexenyl residues may replace the phenyl group of phenylalanine; each such substitution gives rise to a compound that can act as a substrate for the phenylalanyl-sRNA synthetase, but in all cases they are utilized far less readily than the normal substrate.[9] Substitution of a ring proton of the phenyl residue by a slightly larger residue, e.g. by fluorine as in *o*-, *m*- and *p*-fluorophenylalanine, leads to analogues that are accepted by the enzyme (of bacterial, fungal or animal origin) almost as well as phenylalanine itself, and that eventually may be incorporated into protein molecules in place of phenylalanyl residues (see reviews by Richmond[10] and Fowden *et al.*[11]). Similar replacement of a proton by a hydroxy group, as in 3-hydroxyphenylalanine (*m*-tyrosine), a constituent of *Euphorbia* species,[12] results in another growth-inhibitory substance that can become incorporated into the protein of *Bacillus subtilis*.[13]

In extreme instances, the phenyl ring is dispensible for a compound like *trans*-2-aminohex-4-enoic acid with as few as three carbon atom beyond the β-C atom is activated by the phenylalanyl-sRNA synthetase of *E. coli* at approximately 10 per cent of the rate observed for phenylalanine.[9] The slightly larger molecule, *trans*-2-aminohept-4-enoic acid, also serves as a substrate for the enzyme. In both these aliphatic amino acids, the presence of the γ,δ-ethylenic linkage ensures that the β-C atom and all carbons beyond can take up a planar configuration and so resemble an incomplete (and smaller) phenyl ring system. The related 2-amino-4-methylhex-4-enoic acid (II), in which the two methyl groups bear a *cis* configuration about the double bond, has been observed to cause an inhibition of the growth of

[5] R. G. CROUNSE, J. D. MAXWELL and H. BLANK, *Nature* **194**, 694 (1962).
[6] K. T. LIN, J. K. LIN and T. C. TUNG, *J. Formosan Med. Assoc.* **63**, 10 (1964).
[7] P. J. PETERSON and L. FOWDEN, *Biochem. J.* **97**, 112 (1965).
[8] J. EDELSON, P. R. PAL, C. G. SKINNER and W. SHIVE, *J. Am. Chem. Soc.* **78**, 5116 (1956).
[9] T. W. CONWAY, E. M. LANSFORD and W. SHIVE, *J. Biol. Chem.* **237**, 2850 (1962).
[10] M. H. RICHMOND, *Bacteriol. Rev.* **26**, 398 (1962).
[11] L. FOWDEN, D. LEWIS and H. TRISTRAM, *Advan. Enzymol.* **29**, 89 (1967).
[12] K. MOTHES, H. R. SCHÜTTE, P. MÜLLER, M. V. ARDENNE and R. TÜMMLER, *Z. Naturforsch.* **19b**, 1161 (1964).
[13] J. N. ARONSON and G. R. WERMUS, *J. Bacteriol.* **90**, 38 (1965).



(II)

Leuconostoc dextranicum, which can be reversed specifically by addition of phenylalanine or leucine to the growth medium,[14] but it does not seem to act as a substrate for the phenylalanyl-sRNA synthetase from *E. coli*.[9] We have included this last amino acid among the phenylalanine analogues studied because recently it has been shown to represent the principal component of the free amino acid pool of *Aesculus californica* seeds.[15]

In contrast, present information concerning the tyrosyl-sRNA synthetase suggests that it requires in its amino acid substrate a complete planar ring system bearing a hydroxyl group *para* to the alanyl side-chain: the transposition of the hydroxyl group from the *p*- to the *m*-position, as in *m*-tyrosine, produces a phenylalanine and not a tyrosine analogue. Compounds that are effective substrates for enzyme preparations from either *E. coli*[16] or *B. subtilis*[17] include 3-fluorotyrosine, 3-hydroxytyrosine (3,4-dihydroxyphenylalanine) and β-5-hydroxypyridin-2-ylalanine. 3-Hydroxytyrosine was not utilized by a purified preparation of tyrosyl-sRNA synthetase from hog pancreas,[18] and mimosine was inert as a substrate for the *E. coli* enzyme.[16]

In most investigations concerned with the amino acid specificity of the phenylalanyl- and tyrosyl-sRNA synthetases, the ability of the enzymes to utilize analogues has been assayed by either the ATP-PP_i exchange or hydroxamate techniques, i.e. by methods measuring the enzymes' capacity to catalyse the first of the two reactions:

$$\text{amino acid} + \text{ATP} + \text{enzyme} \rightleftharpoons [\text{aminoacyl-AMP-enzyme}] + PP_i$$

$$[\text{aminoacyl-AMP-enzyme}] + \text{tRNA} \rightarrow \text{aminoacyl-tRNA} + \text{AMP}$$

Radioactively-labelled amino acid substrates are required to study the transfer of the aminoacyl group to tRNA and so the alternative technique measuring the formation of aminoacyl-tRNA derivatives has not been used extensively in studies concerned with analogue activations. However, the transfer of labelled 3-fluorotyrosine and *p*-fluorophenylalanine to the corresponding tRNA molecules has been demonstrated.[17, 19] In the present investigation, the ability of phenylalanine analogues to reduce the transfer of ^{14}C-phenylalanine to tRNA has been measured.

RESULTS

Enzyme Fractionation

Aminoacyl-sRNA synthetases were prepared from seed meal of *Phaseolus aureus* (mung bean) or from seeds (minus testas) of *Leucaena leucocephala*, previously soaked overnight in water. The scheme of enzyme purification involved (i) the discarding of protein precipitated from the original extracts after adjusting to pH 6·0, (ii) ammonium sulphate fractionation of the remaining soluble protein, and (iii) the use of DEAE-cellulose columns to further separate

[14] J. EDELSON, C. G. SKINNER, J. M. RAVEL and W. SHIVE, *J. Am. Chem. Soc.* **81**, 5150 (1959).
[15] L. FOWDEN and A. SMITH, *Phytochem.* **7**, 809, (1968).
[16] J. M. RAVEL, M. N. WHITE and W. SHIVE, *Biochem. Biophys. Res. Commun.* **20**, 352 (1965).
[17] R. CALENDER and P. BERG, *Biochemistry* **5**, 1690 (1966).
[18] R. S. SCHWEET and E. H. ALLEN, *J. Biol. Chem.* **233**, 1104 (1958).
[19] H. R. V. ARNSTEIN and M. H. RICHMOND, *Biochem. J.* **91**, 340 (1964).

the enzymes present in the 48·5–55 per cent (*Phaseolus*) or 48·5–60 per cent (*Leucaena*) saturated ammonium sulphate fractions. The activity of the enzyme preparations was measured by an ATP-PP_i exchange technique based on those of DeMoss and Novelli,[20] and Peterson and Fowden.[7]

The pattern of enzyme fractionation obtained with the mung bean extract is illustrated by the data in Table 1. If the original seed extract was not dialysed, the concentration of

TABLE 1. THE STEPWISE PURIFICATION OF THE PHENYLALANYL-sRNA SYNTHETASE FROM MUNG BEAN SEEDS

Fraction	Total protein (mg)	Specific* activity (units)	Relative purification	Enzyme recovery (%)
Homogenate (dialysed)	2095	0·168	1	100
pH 6·0 supernatant, 48·5–55% satd.	1100	0·322	2	101
Ammonium sulphate fraction	29	2·934	17	24
DEAE-cellulose fraction	3	11·22	67	10

* Specific activities are expressed as μmoles PP_i exchanged/hr/mg protein.

Reaction mixtures (final volume 1 ml, pH 7·9) contained tris-HCl buffer (100 μmoles), ATP (4 μmoles), $MgCl_2$ (10 μmoles), ^{32}P-pyrophosphate (4 μmoles, 0·5 μc), phenylalanine (1 μmole), and enzyme. Reaction was for 15 min at 37°.

endogenous amino acids was sufficient to saturate the phenylalanyl-sRNA synthetase present, i.e. no stimulation of ATP-PP_i exchange above the control values was observed when phenylalanine was added to reaction mixtures. When the pH of the original seed extract was lowered from 7·4 to 6·0, about half the protein was precipitated. Seemingly

TABLE 2. THE PROGRESSIVE PURIFICATION OF THE PHENYLALANYL-sRNA SYNTHETASE FROM MUNG BEAN SEEDS

Amino acid	Homogenate (dialysed)	Specific activity* 48·5–55% satd. ammonium sulphate fraction	DEAE-cellulose fraction
Phenylalanine	100	100	100
Cysteine	240	94	41
Glutamic acid	64	9	7
Histidine	192	173	75
Isoleucine	318	27	6
Leucine	359	55	22
Lysine	59	35	11
Proline	141	66	83
Serine	17	23	29
Threonine	132	14	3
Tyrosine	64	39	12
Valine	241	17	7

* Specific activities determined with each amino acid are expressed on the basis of phenylalanine ≡ 100 at each stage.

Reaction mixtures as in Table 1, except amino acids (2·5 μmoles) and tyrosine (0·1 ml of a saturated solution).

[20] J. A. DEMOSS and G. D. NOVELLI, *Biochim. Biophys. Acta* **22**, 49 (1956).

protein discarded at this stage contained little phenylalanyl-sRNA synthetase activity, although values calculated for enzyme recoveries during the early stages of purification tend to be unreliable. The two subsequent purification steps lead to larger proportionate increases in the specific activity of the enzyme, but the recoveries were lower. Finally, a 67-fold increase in the specific activity of the phenylalanyl-sRNA synthetase was achieved.

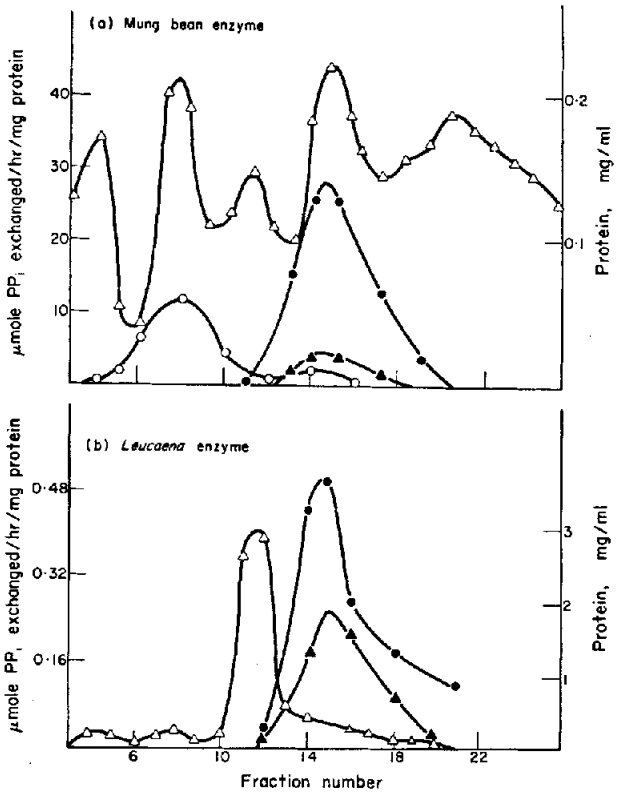


FIG. 1*a*. ILLUSTRATES THE FRACTIONATION OF AMINOACYL-sRNA SYNTHETASE FROM MUNG BEAN ON A DEAE-CELLULOSE COLUMN. THE ACTIVATION OF MIMOSINE BY THE PHENYLALANYL-sRNA SYNTHETASE IS CLEARLY INDICATED.

△ —— △, protein in fractions. Amino acid stimulated ATP-PP_i exchange rates are represented as: ○ —— ○, tyrosine; ● —— ●, phenylalanine; ▲ —— ▲, mimosine.

FIG. 1*b*. ILLUSTRATES THE FRACTIONATION OF PHENYLALANYL-sRNA SYNTHETASE ON A DEAE CELLULOSE COLUMN AND THE ABILITY OF THE ENZYME TO ACTIVATE MIMOSINE.

Key as in Fig. 1*a*.

When a similar fractionation was applied to the *Leucaena* seed extract, the overall degree of purification achieved was much lower. The specific enzymic activity measured after ammonium sulphate fractionation was less than that of enzyme measured after the previous stage, suggesting either an instability of *Leucaena* enzyme in concentrated ammonium sulphate solutions or an incomplete precipitation of enzyme even at 60 per cent saturation ammonium sulphate. The absolute specific activity determined for the most active fraction obtained from the DEAE-cellulose column (0·47 μmoles PP_i exchanged/hr/mg protein) was less than one-twentieth that of the corresponding preparation of phenylalanyl-sRNA synthetase from *P. aureus*.

The most active preparations of *Phaseolus* phenylalanyl-sRNA synthetase used in later experiments were contaminated with other synthetases. Synthetases catalysing histidine- and proline-stimulated ATP-PP_i exchange were most active, exhibiting 75 and 83 per cent respectively of the activity measured with phenylalanine. Table 2 shows the progressive enrichment of the phenylalanyl-enzyme throughout the fractionation steps. Although the phenylalanyl-sRNA synthetase preparations were able to activate tyrosine at about 12 per cent of the rate determined for phenylalanine, the elution pattern from a DEAE-cellulose column (Fig. 1) indicated that the main peaks of phenylalanyl- and tyrosyl-sRNA synthetases were clearly separated. The activation of tyrosine observed with fraction numbers 12–17 was due either to the presence of two distinct tyrosyl-sRNA synthetases in the ammonium sulphate fraction applied to the DEAE-cellulose column or the fact that the phenylalanine activating enzyme shows some affinity for tyrosine. This second alternative seems more likely having regard for the findings with other phenylalanine analogues (see below).

Properties of the Synthetases

pH *optima and stability.* The phenylalanyl-sRNA synthetase from mung bean exhibited an optimum pH of 8·0 under the conditions of assay. The pH-activity curve was not symmetrical, the activity of the synthetase decreasing more sharply on the acid side of the optimum.

The enzyme could be stored in tris-HCl buffer (pH 7·6) at 4° overnight without significant loss of activity; storage for 1 week under these conditions lead to a 25 per cent loss of activity. In contrast, the enzyme lost 90 per cent of its activity when frozen at −40° for 1 week. Addition of thioglycollate (5 mM) had no influence on stability during storage at 4°, but reduced inactivation occurring at −40° to 35 per cent.

Amino acid substrate specificity. (i) The ATP-PP_i exchange reaction. The abilities of the phenylalanyl- and tyrosyl-sRNA synthetases from mung bean to utilize mimosine and some other structural analogues are illustrated in Fig. 1*a* and Tables 3 and 4. It is important to

TABLE 3. ACTIVATION OF PHENYLALANINE, TYROSINE AND SOME ANALOGUES BY MUNG BEAN AMINOACYL-sRNA SYNTHETASES PRESENT IN VARIOUS FRACTIONS ELUTED FROM A DEAE-CELLULOSE COLUMN

	Activation rates for amino acids*					Calculated ratio of exchange rates			
Fraction	Tyr	3Ftyr	Phe	*p*Fphe	Mim	3Ftyr / Tyr	*p*Fphe / Phe	Mim / Phe	Mim / Tyr
48·5–55% $(NH_4)_2SO_4$ fraction applied to DEAE-cellulose	1·10	0·23	4·39	2·62	0·60	0·21	0·60	0·13	0·55
DEAE fraction number									
9	1·74	0·63	—	—	0	0·36	—	—	0
12	12·6	3·56	—	—	0	0·28	—	—	0
15	1·72	0·48	—	—	0·24	0·28	—	—	0·14
18	1·89	0·15	27·0	20·0	3·19	0·08	0·74	0·12	1·70
21	0·53	0	10·9	9·4	1·67	0	0·87	0·15	3·10

Reaction mixtures as in Table 1, except amino acids added separately, tyrosine (1 μmole), 3-fluorotyrosine (1 μmole), mimosine (1 μmole), phenylalanine (2·5 μmoles), *p*-fluorophenylalanine (2·5 μmoles).

* Activation values are expressed as μmoles PP_i exchanged/hr/mg protein. Abbreviations used: tyr, tyrosine; 3Ftyr, 3-fluorotyrosine; phe, phenylalanine; *p*Fphe, *p*-fluorophenylalanine; mim, mimosine.

TABLE 4. THE ABILITY OF VARIOUS ANALOGUES TO STIMULATE ATP-PP_i EXCHANGE IN THE PRESENCE OF THE PHENYLALANYL-sRNA SYNTHETASE FROM MUNG BEAN

Substrate†	Percentage exchange rate* (phenylalanine = 100)
p-Fluorophenylalanine	63
o-Fluorophenylalanine	61
Tyrosine	8
3-Hydroxyphenylalanine	27
Mimosine	14
3,4-Dihydroxyphenylalanine	0
2-Amino-4-methylhex-4-enoic acid	114
2-Amino-4-methylhexanoic acid	3

* Exchange rates were calculated as μmoles PP_i exchanged/hr/mg protein and expressed as percentages of the phenylalanine rate.

† All at 5·0 μmoles/ml except tyrosine, where a saturated solution was used, and mimosine where 2·5 μmoles/ml was used.

Reaction mixtures as in Table 1 except amino acid concentrations as above.

notice that the results shown in Fig. 1*a* and Table 3 relate to synthetase preparations obtained from two different fractionations on DEAE-cellulose, and that there is not a strict correspondence of fraction numbers with peak enzyme activities in the two experiments.

The results illustrated in Fig. 1*a* clearly establish that mimosine stimulates ATP-PP_i exchange in the presence of the phenylalanyl-, but not the tyrosyl-enzyme, and this is confirmed by the approximate constancy of the mimosine/phenylalanine-stimulated exchange rates measured for different DEAE-cellulose fractions (and the very variable ratios for mimosine/tyrosine rates). As expected for two substrates of the same enzyme, the co-addition of mimosine to reaction mixtures containing sufficient phenylalanine to saturate the enzyme did not cause an increase in the rate of ATP-PP_i exchange.

In a similar way, *p*-fluorophenylalanine and 3-fluorotyrosine are indicated as substrates for the phenylalanyl- and tyrosyl-sRNA synthetases respectively by the constancy of the fluorophenylalanine/phenylalanine (fraction No. 18–21, Table 3) and fluorotyrosine/tyrosine (fraction No. 9–15, Table 3) exchange rates. Although the fractions showing peak activity for phenylalanyl-sRNA synthetase always activated tyrosine at a slower rate, fluorotyrosine was not normally activated by these fractions. This observation indicates that tyrosine was acting as a true substrate for the phenylalanyl-sRNA synthetase, and was not being activated by a contaminating tyrosyl-enzyme, which also would have utilized the fluorotyrosine.

In Table 4, the ATP-PP_i exchange rates measured for the mung bean phenylalanyl-sRNA synthetase in the presence of a variety of analogues are recorded. Most substrates are present at concentrations saturating the enzyme, and so the rates measured represent V_{max} values: however, this is not so for mimosine and tyrosine which have low solubilities. 3-Hydroxyphenylalanine (*m*-tyrosine) apparently acts as a better substrate than tyrosine for this enzyme, but it was not activated by the fractions forming the tyrosyl-sRNA synthetase peak in Fig. 1*a*. *o*-Fluorophenylalanine was utilized as a substrate as efficiently as the *p*-fluoro derivative. The most striking feature is that the V_{max} value determined for 2-amino-4-methylhex-4-enoic acid was higher than that for phenylalanine itself, although K_m values determined for the two substances show phenylalanine to have an affinity for the enzyme some forty times greater than that of the analogue (K_m values were 2×10^{-5} and 8×10^{-4} M

respectively). Saturation of the ethylenic linkage of 2-amino-4-methylhex-4-enoic acid, leading to the production of a non-planar molecule (2-amino-4-methylhexanoic acid, homoisoleucine), was associated with an almost complete loss of substrate activity.

Figure 1*b* illustrates the fractionation of the phenylalanyl-sRNA synthetase from *Leucaena*: the eluting buffer contained a steeper KCl concentration gradient than that used with mung bean enzyme. When mimosine was used as a substrate, there was again close coincidence of the activity peaks for the phenylalanine- and mimosine-stimulated reactions, coupled with fairly constant ratios of the mimosine/phenylalanine exchange rates, indicating that phenylalanyl-sRNA synthetase could utilize mimosine effectively. The higher values determined for the ratios (about 0·4 compared with 0·12–0·15 for mung bean enzyme) may partially reflect the fact that the smaller number of *Leucaena* enzyme molecules were more effectively saturated by mimosine at the concentration limit imposed by its low solubility.

(ii) Aminoacyl transfer to tRNA. Initially, the conditions under which the phenylalanyl-sRNA synthetase of mung bean catalysed the transfer of ^{14}C-phenylalanine to a mung bean tRNA preparation were examined. The experimental design adopted ensured that the amount of tRNA in incubation mixtures was limiting, so that any competition by an analogue in the aminoacyl transfer process would be reflected in a reduced formation of ^{14}C-phenylalanyl-tRNA (assayed by the method of Mans and Novelli[21]).

TABLE 5. THE EFFECT OF PHENYLALANINE ANALOGUES ON THE PROCESS OF ^{14}C-PHENYLALANINE TRANSFER TO tRNA CATALYSED BY A MUNG BEAN ENZYME

Substrate	^{14}C-Phenylalanine transfer (counts per 100 sec in tRNA in excess of endogenous value)
Phenylalanine (0·07 μmoles)	987
Phenylalanine + *p*-fluorophenylalanine (20 μmoles)	87
Phenylalanine + 3-hydroxyphenylalanine (20 μmoles)	283
Phenylalanine + mimosine (4 μmoles)	983
Endogenous (i.e. phenylalanine without added tRNA)	278

Reaction mixtures (final volume, 1 ml, pH 8·0) contained tris-HCl buffer (75 μmoles), ATP (5 μmoles), $MgCl_2$ (5 μmoles), EDTA (1 μmole), ^{14}C-phenylalanine (0·5 μc), tRNA (4 mg) and enzyme (DEAE-cellulose fraction). Reaction was for 10 min at 37°.

Under the assay conditions used in the experiments (see Table 5), the formation of ^{14}C-phenylalanyl-tRNA reached a maximum value after a 10 min incubation period. After this time, a progressive slow decrease in the quantity of ^{14}C-phenylalanyl-tRNA in mixtures was observed, presumably reflecting saturation of the available $tRNA^{phe}$ on the one hand coupled with the action of a slight ribonuclease contaminant in the synthetase enzyme preparation. Ribonuclease was employed to confirm that ^{14}C-phenylalanine in the product assayed was attached to RNA.

The transfer of ^{14}C-phenylalanine to tRNA was inhibited by *p*-fluorophenylalanine (>90 per cent) and by *m*-tyrosine (>70 per cent) at high analogue concentrations (about 290 times that of phenylalanine); in contrast, when mimosine was employed at a concentration sixty times that of ^{14}C-phenylalanine, it caused no inhibition of ^{14}C-phenylalanine transfer (Table 5). Presumably a small quantity of tRNA may have been present in the enzyme

[21] R. J. MANS and G. D. NOVELLI, *Arch. Biochem.* **94**, 48 (1961).

preparation to account for the ^{14}C-phenylalanine transfer (endogenous) observed in the absence of added tRNA; alternatively a small quantity of ^{14}C-phenylalanine may be absorbed onto the protein precipitated during the assay.

DISCUSSION

During the fractionation of the aminoacyl-sRNA synthetases from mung bean, the activities of almost all the enzymes were reduced in comparison to that of the phenylalanyl-enzyme at each step. The leucyl-, isoleucyl- and valyl-sRNA synthetases were the most active in the dialysed homogenate of seeds, but the activity of each of these enzymes had been reduced to a low level in the 48·5–55 per cent saturated ammonium sulphate fraction. The low initial activity of the seryl-enzyme tended to separate with the phenylalanine activating enzyme at each stage, and the DEAE-cellulose treatment obviously concentrated the prolyl-sRNA synthetase along with the desired phenylalanyl-enzyme. Probably several additional fractionation steps would be required to produce a homogenous preparation of the phenylalanyl-sRNA synthetase.

Within the range of amino acid analogues tested, the substrate specificities of the phenylalanyl- and tyrosyl-sRNA synthetases from mung bean resembled those of animal and bacterial enzymes. However, the activation of mimosine by the phenylalanyl-enzyme is a new observation, for previously it only seems to have been considered and tested as a substrate for the tyrosyl-sRNA synthetase with negative results.[16] In a similar way, the observation that tyrosine probably acts as a poor substrate for the plant phenylalanyl-sRNA synthetase appears to be novel, although a report exists of the reverse situation in which phenylalanine was activated by the tyrosyl-enzyme from hog pancreas.[22] Here, the K_m calculated for phenylalanine (2×10^{-2} M) indicated that the tyrosyl-enzyme exhibited a low order of affinity for the anomalous substrate. In these last two instances the anomalous amino acid is unlikely to be transferred from the adenylate complex to tRNA (compare the instance of the formation of a valyladenylate complex catalysed by isoleucyl-sRNA synthetase, and its subsequent breakdown to regenerate valine in which $tRNA^{isol}$ appears to play a specific catalytic role[23, 24]). In general, it appears that small substituents may replace protons on the phenyl ring at the *o*-, *m*- or *p*-positions of phenylalanine without complete loss of substrate activity in respect of mung bean phenylalanyl-sRNA synthetase. However the replacement of two adjacent protons as seen in 3,4-dihydroxyphenylalanine presumably produced a molecule too large to be accommodated at the active centre of the enzyme.

The relative efficiencies with which mimosine, *p*-fluorophenylalanine and *m*-tyrosine are used as substrates for the mung bean phenylalanyl-sRNA synthetase cannot be correlated with the growth inhibitory effects that they cause in mung bean seedlings. Mimosine is easily the most potent growth inhibitor, seedlings being killed by levels of about 1 mg/g dry weight, but it forms a relatively poor substrate for the activating enzyme, and has no measurable effect upon the transfer of phenylalanine to tRNA. Therefore it seems unlikely that mimosine-induced growth inhibitions result from a restriction of phenylalanine incorporation into protein or by the anomalous insertion of mimosine residues into protein molecules in place of those of phenylalanine. This idea is supported by the observation that mimosine stimulates ATP-PP_i exchange in the presence of the phenylalanyl-sRNA synthetase from *Leucaena*, a

[22] J. M. Clark and J. P. Eyzaguirre, *J. Biol. Chem.* **237**, 3698 (1962).
[23] A. N. Baldwin and P. Berg, *J. Biol. Chem.* **241**, 831, 839 (1966).
[24] R. B. Loftfield and E. A. Eigner, *Fed. Proc.* **23**, 164 (1964).

species that elaborates large amounts of mimosine, but whose growth is not inhibited by mimosine. Initially, it had seemed that the phenylalanyl-sRNA synthetase from different species might exhibit variable amino acid substrate specificity and so be comparable to the prolyl-sRNA synthetases from mung bean and liliaceous plants: in this last case, the prolyl-enzyme from liliaceous plants that produce azetidine-2-carboxylic acid did not activate this toxic substance, whereas the corresponding enzyme from mung bean could not discriminate against the proline analogue.[7]

The activation of 2-amino-4-methylhex-4-enoic acid by the phenylalanyl-sRNA synthetase of mung bean raises the same question of substrate specificity of enzymes from different species. Here, the analogue is a major component of developing *Aesculus californica* seeds, being laid down in the seed during a period of active protein synthesis. Therefore it will be interesting to learn whether the phenylalanyl-enzyme from *Aesculus* fails to activate this phenylalanine analogue or, if an aminoacyladenylate complex is formed, how the further transfer of the aminoacyl residue to $tRNA^{phe}$ is limited. This problem will form the subject of a separate investigation.

EXPERIMENTAL

Materials

Plant materials. Seed of *Phaseolus aureus* was ground in a laboratory mill to pass through a 0·5 mm mesh sieve. The *Leucaena leucocephala* seed (supplied through the Plant Introduction Section, C.S.I.R.O., Canberra) was chipped to facilitate imbibition of water and soaked overnight: the testas were removed before use.

Amino acids. The L-isomers of the normal protein amino acids, *o*- and *p*-fluoro-, 3-hydroxy- and 3,4-dihydroxy-phenylalanines were high-grade commercial samples. Mimosine was isolated from *L. leucocephala* seed and 2-amino-4-methylhex-4-enoic acid from seed of *Aesculus californica*. 2-Amino-4-methylhexanoic acid was prepared by catalytic hydrogenation of 2-amino-4-methylhex-4-enoic acid using Adam's PtO_2 catalyst. 3-Fluorotyrosine was the gift of Dr. W. Shive (Austin, Texas).

Radioisotopic chemicals. L-[U-^{14}C]Phenylalanine (7·1 μc/μmole) was obtained from the Radiochemical Centre, Amersham.

[^{32}P]Pyrophosphate was prepared from sodium [^{32}P]orthophosphate (Radiochemical Centre) by pyrolysis to give a product of specific activity 0·125 μc/μmole.

Methods

Determination of protein. The method of Lowry *et al.*[25] was normally adopted using bovine serum albumin as a standard: when dilute protein solutions were assayed, corrections had to be made for interference by tris-HCl present in the buffers. This method was unsuitable for use with fractions eluted from DEAE-cellulose columns, which contained high concentrations of KCl, and protein contents were calculated from extinction values measured at 260 and 280 nm (see Layne[26]).

Enzyme fractionations. All operations were carried out in a cold room at 1–4°. The following steps were used for the mung bean enzyme preparations:

(*a*) Homogenate and pH 6·0 supernatant fraction. Seed meal (25 g) was ground manually with 0·1-M-tris-HCl buffer, pH 7·6 (65 ml), containing 0·4 M-sucrose. The macerate was pressed through muslin and centrifuged at 12,000 *g* for 15 min. An aliquot of the supernatant was dialysed to give the dialysed homogenate fraction. The remainder was adjusted to pH 6·0 by careful addition of 0·2 N-acetic acid with constant stirring. Protein precipitated was discarded and the residual supernatant represented the pH 6·0 supernatant fraction. It was readjusted to pH 7·9 before assay or further fractionation.

(*b*) Ammonium sulphate fractionation. Ammonium sulphate was added to the pH 6·0 supernatant fraction to give 48·5 per cent saturation (calculated for 0°) and protein precipitation was allowed to proceed for 15 min. The sedimented protein (12,000 *g*, 15 min) was discarded and further ammonium sulphate added to the supernatant to give 55 per cent saturation. The protein precipitated at this stage was collected by centrifuging and redissolved in 0·1 M-tris-HCl buffer, pH 7·9 (4 ml), using an all-glass homogenizer. Residual ammonium sulphate was removed by dialysis against 0·1 M-tris-HCl buffer, pH 7·6 (twice changed during a 3 hr dialysis period).

[25] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[26] E. LAYNE, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 3, p. 447. Academic Press, New York (1957).

(*c*) DEAE-cellulose fractionation. A small column (10 × 2 cm dia.) was packed with pre-swollen Whatman DE 52 cellulose suspended in 0·1 M-tris-HCl buffer, pH 7·6. The dialysed ammonium sulphate fraction was added and then the column was eluted with a linear salt gradient obtained by mixing 0·1 M-tris-HCl buffer, pH 7·6 (70 ml), and buffer (70 ml) containing 0·2 M-KCl. Fractions (5–7 ml) were collected and alternate ones were assayed for aminoacyl-sRNA synthetase activity by the ATP-PP_i exchange technique. For further studies with the phenylalanyl-sRNA synthetase, the three fractions containing maximum enzyme activity were combined.

The procedure used for the isolation of the phenylalanyl-sRNA synthetase from *Leucaena* seeds was based on that outlined above for *P. aureus* enzyme, with the following modifications: (i) a wider range ammonium sulphate fraction was used, i.e. the 48·5–60 per cent saturation protein precipitate, and (ii) a steeper salt gradient was employed for eluting the DEAE-cellulose column, 0·1 M-tris-HCl buffer, pH 7·6 (70 ml), being mixed with buffer (70 ml) containing 0·4 M-KCl.

tRNA *isolation.* The general phenol method of Holley *et al.*[27] was employed to extract tRNA from mung bean seed flour (75 g) as previously described.[7] The tRNA fraction precipitated by ethanol was applied in 0·05 M-tris-HCl buffer, pH 7·5 (15 ml), to a small DEAE-cellulose column (5 × 2 cm dia.). The column was washed with more buffer (100 ml) and then tRNA was displaced with M-NaCl. Fractions exhibiting strong extinction at 260 nm were treated with 3 volumes of ethanol to precipitate tRNA, which was twice washed with 85 per cent ethanol and twice with ether before drying in a vacuum. All operations were performed at 1–4°; yield, 50 mg.

Assay procedures. (*a*) ATP-PP_i exchange system. The incubation mixtures generally contained: tris-HCl buffer (100 μmoles), $MgCl_2$ (10 μmoles), ATP (4 μmoles), [^{32}P]pyrophosphate (4 μmoles), amino acid (5 μmoles) and enzyme preparation; total volume, 1 ml. Amino acid concentrations were varied in some experiments dependent upon the availability or solubility of individual compounds and in others designed to measure K_m values. In most instances, the pH of the final incubation mixture was 7·9.

Reactions were allowed to proceed for 15 min at 37°, and then stopped by plunging the reaction tubes into ice and adding 7·5 per cent (w/v) trichloroacetic acid (2 ml). Precipitated protein was removed by centrifuging and an aliquot (1 ml) of supernatant was added to charcoal (50 mg) suspended in 0·1 M-sodium acetate, pH 5·0 (4·5 ml). The mixture was shaken periodically during 30 min to complete the adsorption of the ATP onto charcoal, which then was filtered and layered uniformly on to a disc of filter paper supported on a specially designed stainless-steel planchet. Radioactivity in the adsorbed ATP was assayed using a thin end-window Geiger-Müller tube attached to an Isotope Development Limited 1700 scaler.

The exchange rates were calculated following the method of Davie *et al.*[28]

(*b*) Assay of phenylalanyl-tRNA formation. Incubation mixtures contained: $MgCl_2$ (5 μmoles), ATP (5 μmoles), EDTA (1 μmole), ^{14}C-phenylalanine (0·07 μmoles, 0·5 μc), tris-HCl buffer, pH 7·5 (75 μmoles), tRNA preparation (4 mg) and enzyme preparation; total volume, 1 ml. Reactions proceeded for 10 min at 37° and were stopped by mixing with a 2 per cent casein in N-KOH solution (0·3 ml). An aliquot (0·3 ml) of this mixture was absorbed into a disc of filter paper and "fixed" by treatment with 10 per cent (w/v) trichloroacetic acid. The further steps used for the determination of ^{14}C-phenylalanyl-tRNA followed the method of Mans and Novelli,[21] radioactivity finally being determined by scintillation counting (Isotope Development Limited liquid measuring head 2022).

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[28] E. W. Davie, V. V. Koningsberger and F. Lipmann, *Arch. Biochem.* **65**, 21 (1956).