Phytochemistry, 1972, Vol. 11, pp. 2129 to 2138. Pergamon Press. Printed in England.

STEREOSPECIFICITY OF GLUTAMYL-*t*RNA SYNTHETASE ISOLATED FROM HIGHER PLANTS

P. J. LEA and L. FOWDEN

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

(*Received* 19 *January* 1972)

Key Word Index—*Phaseolus aureus*; *Caesalpinia bonduc*; Leguminosae; *Hemerocallis fulva*; Liliaceae; Glutamyl-*t*RNA synthetase; γ-substituted glutamic acids; amino acid stereospecificity.

Abstract—The amino acid substrate specificity of glutamyl-*t*RNA synthetase, prepared from *Phaseolus aureus* seed and two plant materials (*Hemerocallis fulva* leaves and *Caesalpinia bonduc* seed) containing γ-substituted glutamic acids, have been examined. The enzyme from *P. aureus* activated a range of γ-substituted glutamic acids. The enzymes from analogue-producing plants have developed a mechanism which prevents the activation of their constituent analogues. Optimum assay conditions, sensitivity to salts, and the general stability of the enzyme are also described.

INTRODUCTION

GLUTAMYL-*t*RNA synthetase has been isolated and purified from yeast,[1] *Escherichia coli*,[2] pig liver[3] and rat liver.[4] Generally, very high K_m values were determined for glutamate when the ATP-PPi exchange assay technique was used, but the addition of *t*RNA greatly reduced the concentration of glutamate necessary to give maximum activation.[3,4] The unusually high K_m values may explain why almost all studies of the levels of aminoacyl-*t*RNA synthetases in plants, measured by the ATP–PPi exchange method, have recorded either low or zero activities of the glutamyl enzyme.[5–8] Only Atwood and Cocking,[9] using the hydroxamate assay, recorded high activities relative to other aminoacyl-*t*RNA synthetases for the glutamyl enzyme in tomato roots. Failure to detect glutamyl-*t*RNA synthetase activity in many plant extracts is difficult to rationalize with the high content of glutamic acid in many plant proteins.[10]

Higher plants synthesize a great variety of amino acids, and a recent count places their number at about 200.[11] Certain of these amino acids behave as analogue molecules, interacting with processes concerned in the metabolism of individual members of the group of 20 protein amino acids.

[1] N. COLES and A. MEISTER, *Proc. Natl. Acad. Sci. U.S.A.* **48,** 1602 (1962).
[2] R. A. LAZZARINI and A. H. MEHLER, *Biochem.* **3,** 1445 (1964).
[3] L. W. LEE, J. M. RAVEL and W. SHIVE, *Arch. Biochem. Biophys.* **121,** 614 (1967).
[4] M. P. DEUTSCHER, *J. Biol. Chem.* **242,** 1123 (1967).
[5] E. MOUSTAFA and J. W. LYTTLETON, *Biochim. Biophys. Acta* **68,** 45 (1963).
[6] J. D. HENSHALL and T. W. GOODWIN, *Phytochem.* **3,** 677 (1964).
[7] J. W. ANDERSON and L. FOWDEN, *Plant Physiol.* **44,** 60 (1969).
[8] A. LEGOCKI, E. M. MACKOWIAK and J. PAWELKIEWICZ, *Bull. l'Acad. Polon Sci.* **15,** 435 (1967).
[9] M. M. ATWOOD and E. C. COCKING, *Biochem. J.* **96,** 616 (1965).
[10] N. L. KENT, in *Proteins as Human Food* (edited by R. A. LAWRIE), p. 280, Butterworth, London (1970).
[11] L. FOWDEN, in *Progress in Phytochemistry* (edited by L. REINHOLD and Y. LIWSCHITZ), Vol. 2, p. 203, Wiley, London (1970).

Investigations in the authors' laboratories have provided data concerning the amino acid substrate specificities of prolyl-,[12] arginyl-[13] and phenylalanyl-[14,15] *t*RNA synthetases, extracted from plant species that produce compounds exhibiting structural analogies with the normal substrate molecules. The specificities of such enzymes are usually more exacting than those of comparable enzymes obtained from plants that do not produce the particular analogue molecules; these latter enzymes often readily accept the analogues as substrates. Apparently, a modified conformation at the active site of particular synthetases has been evolved by species producing such analogues to prevent incorporation of possibly harmful constituents into their cellular proteins.

Analogues of glutamic acid occur in a number of plant species;[11] in particular, γ-substituted glutamic acids have been found in high concentrations in some species. Glutamyl-*t*RNA synthetase was purified from analogue-containing plants: *Hemerocallis fulva*, a liliaceous species producing *threo*-γ-hydroxy-L-glutamic acid (II);[16] and *Caesalpinia bonduc*, a legume whose seed contains large quantities of *erythro*-γ-methyl-L-glutamic acid (IV), and in lesser amounts γ-ethylidene- and γ-ethyl-L-glutamic acids (Watson and Fowden, unpublished). The enzyme also was purified from a seed (*Phaseolus aureus*) from which there is no record of occurrence of any substituted glutamic acids. Two further plants producing substituted derivatives of glutamic acid were examined: (I) *Phyllitis scolopendrium* and (II) *Gleditsia triacanthos*, but only very low levels of glutamyl-*t*RNA synthetase activity could be detected. This paper reports a comparative study of the properties, and in particular the substrate specificities, of glutamyl-*t*RNA synthetases extracted from the first three plant species.

RESULTS

Enzyme Fractionation

Glutamyl-*t*RNA synthetase was purified from the three plant sources according to the scheme outlined in Table 1. Preliminary studies with the glutamyl-*t*RNA synthetase from

TABLE 1. PURIFICATION OF GLUTAMYL-*t*RNA SYNTHETASE FROM THREE PLANT SPECIES

Species	Purification treatment	Recovery of glutamyl-*t*RNA synthetase (% of enzyme activity in crude extract)	Contaminating aminoacyl-*t*RNA synthetase (% of glutamyl-*t*RNA synthetase in each fraction)	Specific activity (nmol ^{32}PPi exchanged/min/mg of protein)	Relative purification
Phaseolus aureus (seed)	Crude extract	100	2800	0·52	1
	$(NH_4)_2SO_4$ (45–55%)	53	710	1·54	2·96
	Sephadex G-25 fraction	34	118	6·1	11·6
	Acetone (60–80%)	28	62	13·3	25·7
	DEAE-cellulose peak	8·5	24	26·3	50·8
Caesalpinia bonduc (seed)	Crude extract	100	3600	0·38	1
	$(NH_4)_2SO_4$ (45–55%)	48	935	0·96	2·53
	Sephadex G-25 fraction	26	248	3·26	11·7
	Acetone (60–80%)	18	131	7·82	20·6
	DEAE-cellulose peak	5·3	62	14·82	38·9
Hemerocallis fulva (leaf)	Crude extract	100	3130	1·4	1
	$(NH_4)_2SO_4$ (40–50%)	42	865	4·0	2·86
	Sephadex G-25 fraction	22	206	18·9	13·5
	Acetone (75–90%)	12	98	39·6	28·2

[12] P. J. PETERSON and L. FOWDEN, *Biochem. J.* **97**, 112 (1965).
[13] L. FOWDEN and J. B. FRANKTON, *Phytochem.* **7**, 1077 (1968).
[14] I. K. SMITH and L. FOWDEN, *Phytochem.* **7**, 1065 (1968).
[15] J. W. ANDERSON and L. FOWDEN, *Biochem. J.* **119**, 677 (1970).
[16] L. FOWDEN and F. C. STEWARD, *Ann. Bot. N.S.* **21**, 53 (1957).

P. aureus indicated that the enzyme was retarded, relative to other synthetases, when extracts were passed through Sephadex G-25 to such an extent that a useful separation could be achieved (Fig. 1). In practice, an ammonium sulphate fractionation step was introduced first to enable a concentrated synthetase preparation to be applied to the Sephadex column. When cold acetone was added to the Sephadex fractions, no protein was precipitated until at least 50% (v/v) acetone had been added. Glutamyl-*t*RNA synthetase activity precipitated as indicated in Table 1; this step also served to concentrate the enzyme before it was applied to the DEAE-cellulose column.

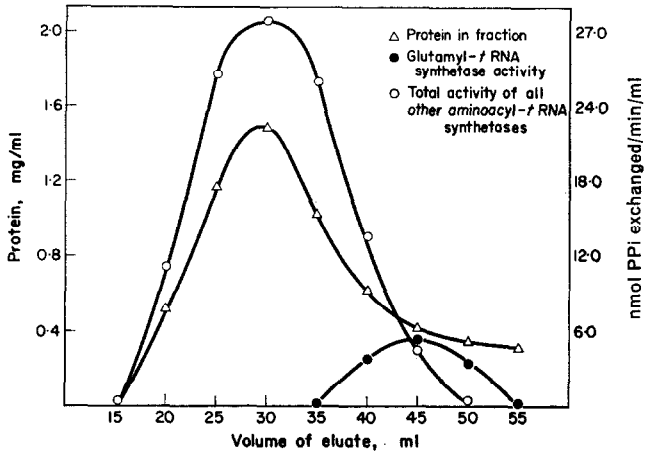


FIG. 1. ELUTION PROFILE OF PROTEIN, GLUTAMYL-*t*RNA SYNTHETASE AND OTHER AMINOACYL-*t*RNA SYNTHETASES FROM A SEPHADEX G-25 COLUMN.

Glutamyl-*t*RNA synthetase extracted from *P. aureus* and *C. bonduc* behaved almost identically during the purification steps, except for a slight difference in position of the enzyme-containing peak eluted from the DEAE-cellulose column (Figs. 2a and b). The

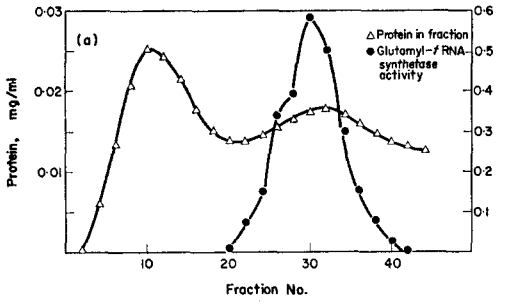


FIG. 2a. FRACTIONATION OF GLUTAMYL-*t*RNA SYNTHETASE FROM *P. aureus* ON A DEAE-CELLULOSE COLUMN.

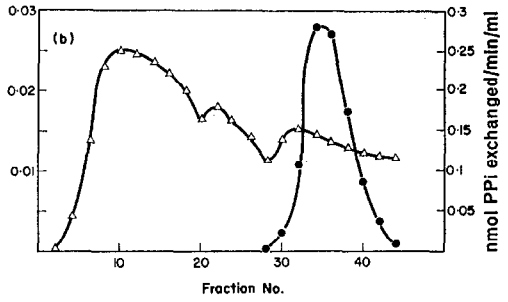


FIG. 2b. FRACTIONATION OF GLUTAMYL-*t*RNA SYNTHETASE FROM *C. bonduc* ON A DEAE-CELLULOSE COLUMN.

purification of enzyme from *H. fulva*, involved the selection of slightly different fractions at each stage; this may have resulted from the much lower concentrations of protein present in leaf extracts. This enzyme was not subjected to a final purification step on DEAE-cellulose, because preliminary studies indicated that virtually all activity was lost during elution from the column. The level of contamination by other synthetases, in the purified extract (Table 2), compares favourably with values obtained for other plant aminoacyl-*t*RNA synthetases.[12,14,15]

TABLE 2. A COMPARISON OF THE ACTIVITY OF INDIVIDUAL AMINOACYL-*t*RNA SYNTHETASES* IN CRUDE AND PURIFIED PLANT PREPARATIONS

Amino acid substrate	*Phaseolus aureus*		*Caesalpinia bonduc*		*Hemerocallis fulva*	
	Crude extract (dialysed)	DEAE—cellulose peak	Crude extract (dialysed)	DEAE—cellulose peak	Crude extract (dialysed)	75–90% acetone fraction
Glutamic acid	100	100	100	100	100	100
Alanine	62	0	85	0	82	2
Arginine	45	0	110	0	30	0
Aspartic acid	55	0	43	0	66	0
Asparagine	65	0	88	0	94	4
Cysteine	226	11·6	316	13	80	0
Glutamine	0	0	0	0	32	0
Glycine	0	0	0	0	40	0
Histidine	241	5·5	536	18	224	24
Isoleucine	865	0	612	4	832	23
Leucine	823	0	234	1	818	18
Lysine	120	0	317	16	156	6
Methionine	51	0	142	0	70	0
Phenylalanine	106	2·5	208	6	181	1
Proline	175	12·6	204	0	90	0
Serine	132	0	202	0	212	12
Threonine	104	0	226	3	163	3
Tryptophan	86	0	162	1	144	4
Tyrosine	70	0	87	0	200	0
Valine	194	0	309	0	328	8

* Values quoted as a percentage of the glutamyl-*t*RNA synthetase activity in each extract, assayed by the ATP–PPi exchange method.

Properties of P. aureus *glutamyl-tRNA synthetase*

Optimum conditions for ATP-PPi exchange assay. The pH optimum was found to be a plateau in the 7·8–8·2 region: a rapid drop in activity was observed at pH values less than 7·0. The optimum temperature and time for the assay was established as 37° for 12 min. K_m values for Mg^{2+} and ATP are given in Table 3. ATP and pyrophosphate were always present in reaction mixtures at equimolar concentrations. The K_m value for glutamic acid was found to be $7{\cdot}2 \times 10^{-3}$ M; this value was unaffected by addition of low concentrations of *t*RNA to reaction mixtures; at concentrations > 0·4 mg/ml, *t*RNA inhibited the exchange reaction. When the concentration of glutamate in reaction mixtures was increased above 100 mM, a decrease in the rate of ATP-PPi exchange was observed; this was at first thought to be substrate inhibition. Further experiments indicated that salts at concentrations above 100 mM inhibited the exchange reaction (Table 4). A study of the effect of various salts

TABLE 3. K_m VALUES * OF ATP AND Mg^{2+} DETERMINED FOR GLUTAMYL-*t*RNA SYNTHETASE BY TWO ASSAY METHODS

Plant species	Assay by ATP-PPi exchange		Assay by ^{14}C-glutamate-*t*RNA formation	
	ATP ($\times 10^4$)	Mg^{2+} ($\times 10^3$)	ATP ($\times 10^4$)	Mg^{2+} ($\times 10^3$)
P. aureus	3·2	1·8	5·3	4·8
C. bonduc	4·5	2·2	7·2	6·3
H. fulva	4·3	3·4	—	—

* Expressed as molar concentrations.

on the exchange reaction was somewhat confused because, (i) 35 μmol/ml of the potassium salt of glutamic acid were normally added to reaction systems, and (ii) the enzyme extract obtained from the DEAE-cellulose column contained approximately 50 mM KCl. Therefore no attempt was made to calculate K_i values, relating to the inhibition caused by other ions in the assay medium.

TABLE 4. A COMPARISON OF THE % INHIBITION OF *P. aureus* GLUTAMYL-*t*RNA SYNTHETASE (MEASURED BY THE ATP–PPi EXCHANGE ASSAY), BY FOUR SALTS TESTED AT TWO CONCENTRATIONS

Salt	Concn in assay medium	
	100 mM	500 mM
KCl	15	62
LiCl	22	73
NH_4Cl	31	82
NaCl	40	91

Optimum conditions for the formation of ^{14}C-*glutamyl-*t*RNA.* The pH optimum was found to be very similar to that described for the exchange assay. At 37° great care was taken to control the incubation time for the assay; the reaction rate was linear up to 6 min, but after 12 min the total amount of ^{14}C-glutamyl-*t*RNA began to decline. The concentration of *t*RNA required was also found to be critical. The incorporation of ^{14}C-glutamate into *t*RNA measured after 6 min, was linear with concentrations up to 0·3 mg/ml; concentrations above 0·5 mg/ml progressively inhibited the transfer of ^{14}C-glutamate to *t*RNA. K_m values for Mg^{2+} and ATP are shown in Table 3; no attempt was made to measure the K_m for glutamate.

Enzyme Stability

The enzyme was unstable, 85% of the activity being lost in 24 hr at −20°. Attempts to stabilize the enzyme, with various sulphydryl reagents, glycerol and glutamic acid were unsuccessful. However, 10% glycerol added to the extraction medium did increase the yield of the enzyme.

Amino Acid Substrate Specificity

A large number of potential glutamic acid analogues (at a concentration of 75 mM) were tested for their ability to promote the ATP–PPi exchange reaction in the presence of purified enzyme extracted from the three species under investigation (Table 5). Analogues

TABLE 5. KINETIC PARAMETERS DETERMINED FOR GLUTAMIC ACID AND SEVERAL OF ITS ANALOGUES USING GLUTAMYL-*t*RNA SYNTHETASE * PREPARATIONS FROM VARIOUS HIGHER PLANTS

Substrate		*Phaseolus aureus* seed	*Hemerocallis fulva* leaf	*Caesalpinia bonduc* seed
L-Glutamic acid	K_m	$7{\cdot}21 \times 10^{-3}$	$5{\cdot}24 \times 10^{-3}$	$9{\cdot}3 \times 10^{-3}$
	V_{max}	100	100	100
erythro-γ-Methyl-L-glutamic acid	K_m	$1{\cdot}55 \times 10^{-2}$	$2{\cdot}81 \times 10^{-2}$	∞
	V_{max}	68·1	40·2	0
threo-γ-Methyl-DL-glutamic acid	K_m	—	∞	—
	V_{max}	55·2 (75 mM)	0	20·1 (75 mM)
threo-γ-Hydroxy-L-glutamic acid	K_m	$2{\cdot}11 \times 10^{-2}$	∞	$5{\cdot}21 \times 10^{-2}$
	V_{max}	54	0	Calc. as 23·6
erythro-γ-Hydroxy-DL-glutamic acid	K_m	—	—	∞
	V_{max}	58·2 (75 mM)	34·2 (75 mM)	0
2(*S*),4(*S*)-γ-Hydroxy-γ-methyl-L-glutamic acid	K_m	$3{\cdot}43 \times 10^{-2}$	$1{\cdot}25 \times 10^{-1}$	∞
	V_{max}	42·2	Calc. as 10·2	0
2(*S*),4(*R*)-γ-Hydroxy-γ-methyl-L-glutamic acid	K_m	—	∞	∞
	V_{max}	38·2 (75 mM)	0	0

The following analogues of glutamic acid were also tested at concentrations of 75 mM, but no pyrophosphate exchange was detected: γ-methylene-DL-glutamic acid, γ-ethyl-L-glutamic acid, γ-ethylidene-L-glutamic acid, γ-ethyl-L-glutamic acid, β-methyl-DL-glutamic acid, β-hydroxy-DL-glutamic acid, DL-α-aminoadipic acid, *cis*-L-α-(carboxycyclopropyl)glycine, *trans*-L-α-(carboxycyclopropyl)glycine.

K_m values are expressed as molar concentrations with respect to the L-form.

V_{max} values are expressed as percentages of the values determined for glutamic acid.

* Assayed by ATP–PPi exchange.

promoting exchange were further tested (if sufficient quantities were available) at varying concentrations and K_m values calculated. Attempts to show competition between glutamic acid and the various analogues for the enzyme, by the ATP-PPi exchange reaction, were unsuccessful, probably because the analogues could be used at a concentration only double that of glutamic acid, before inhibition attributable to K^+ was encountered. However, the rate of ATP–PPi exchange determined with mixtures of an analogue and glutamic acid never exceeded that obtained with glutamic acid alone. Hence it is highly improbable that activation of the γ-substituted glutamic acids was effected by contaminating aminoacyl-*t*RNA synthetases. The direct transfer of analogues to *t*RNA was not investigated, because suitably labelled analogue preparations were not available. However, the assay based on aminoacyl-*t*RNA formation can be used to measure the inhibitory effect of an unlabelled

analogue upon the transfer of ^{14}C-glutamate to *t*RNA. The analogues were added to standard assay mixtures at a concentration 400 times that of the ^{14}C-glutamic acid used, and the results obtained with purified enzymes isolated from *P. aureus* and *C. bonduc* are given in Table 6. Regrettably, leaves of *H. fulva* were not available at the time of these investigations.

TABLE 6. PERCENTAGE INHIBITION OF TRANSFER OF ^{14}C-GLUTAMATE TO *t*RNA BY SUBSTITUTED GLUTAMIC ACIDS

Glutamic acid derivative	*P. aureus*	*C. bonduc*
erythro-γ-Methyl-L-	75·6	0
threo-γ-Methyl-DL-	72·7	58·2
threo-γ-Hydroxy-L-	79·1	65·2
erythro-γ-Hydroxy-DL-	80·2	0
2(*S*), 4(*S*)-γ-Hydroxy-γ-methyl-L-	0	0
2(*S*), 4(*R*) -γ-hydroxy-γ-methyl-L-	0	0

Reaction mixtures contained $4{\cdot}4 \times 10^{-3}$ mM ^{14}C-glutamate and 1·76 mM substituted glutamic acids.

DISCUSSION

For the three plants investigated, the maximum increase in specific activity of the glutamyl-*t*RNA synthetase (50-fold for the *P. aureus* enzyme) was low compared with purifications obtained for other plant aminoacyl-*t*RNA synthetases.[9,12,14,15] The low increase in specific activity obtained was probably attributable in large part to enzyme instability; no other plant synthetase with which we have worked has proved so labile. However, if the substrate specificity of an aminoacyl-*t*RNA synthetase is under investigation, a more important aspect of purification is the removal of contaminating synthetases. If ATP–PPi exchange, attributable to any individual contaminating synthetase, proceeded at a rate higher than that determined for glutamate analogues, it clearly would be impossible to decide with certainty which synthetase enzyme was responsible for activating the analogue. Such problems have arisen in studies on the specificity of arginyl-*t*RNA synthetase in *Canavalia ensiformis*,[13] where homoarginine was activated by a contaminating lysine enzyme. Similarly, norleucine was activated by both methionyl- and leucyl-*t*RNA synthetases isolated from rabbit reticulocytes.[17] However, the maximum contamination of the glutamyl-*t*RNA synthetase preparations by any specific enzyme was 12·6% in *P. aureus*, 18% in *C. bonduc* and 32% in *H. fulva*, as determined by measurement of ATP–PPi exchange rates (see Table 2).

The most important step in the purification of the glutamyl enzyme was the separation achieved on Sephadex G-25. There are three possible reasons for this retardation, (i) the enzyme has a low MW (a survey of aminoacyl-*t*RNA synthetase MWs indicated they all lie between 90 000 and 180 000[18]), (ii) the enzyme splits into subunits, some of which recombine to form an active enzyme after elution from the Sephadex G-25. Subunits have been identified for the methionyl-,[19] seryl-,[20] and prolyl-[21] *t*RNA synthetases, (iii) the enzyme

[17] S. NEALE, *Chem.-Biol. Interactions* **2**, 349 (1970).
[18] P. J. PETERSON. *Biol. Revs.* **42**, 552 (1967).
[19] C. J. BRUTON and B. J. HARTLEY, *Biochem. J.* **108**, 281 (1968).
[20] J. R. KATZE, *Fed. Proc.* **27**, 799 (1968).
[21] M. L. LEE and K. H. MUENCH, *J. Biol. Chem.* **244**, 223 (1969).

exists in the form of a complex which is adsorbed onto Sephadex G-25. Enzyme-AMP-aminoacyl complexes with such adsorption properties have been reviewed recently by Allende and Allende.[22] No attempt has been made to investigate any of these theories due to the low activity and instability of the enzyme.

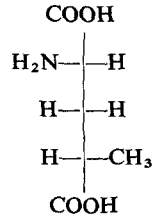
K_m values for glutamate, determined by the ATP–PPi exchange assay for the glutamyl-*t*RNA synthetases from rat liver[4] and *Escherichia coli*[3] were 0·2 and 0·4 M in the absence of *t*RNA, and $6{\cdot}7 \times 10^{-4}$ and 6·6 and 10^{-4} M in the presence of *t*RNA, respectively. Our experiments with plant enzymes showed that the K_m value was not affected by *t*RNA: it is possible that the *t*RNA preparation used was either inactive[23] or contained inhibitors[24] which prevented the increase of ATP–PPi exchange obtained with other materials. However, the normally determined K_m value of $7{\cdot}2 \times 10^{-3}$ M for *P. aureus* is much lower than those obtained for rat liver[4] and *E. coli*,[3] and it seems probable that the plant enzyme really does not require *t*RNA to effect the ATP–PPi exchange reaction. Such a difference between the arginyl-*t*RNA synthetase from plant (*C. ensiformis*)[13] and bacterial (*E. coli*)[23] sources has been noted previously.

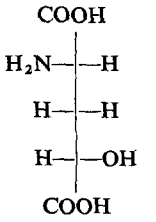
Although values for the K_m of ATP and Mg^{2+} are given, these do not reflect the true situation as complexes, $MgATP^{2-}$ and $MgP_2O_7^{2-}$, are thought to act as the principle substrates for the ATP-PPi exchange reaction.[25]

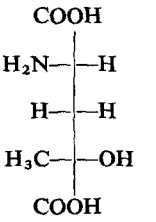
The inhibition of ATP–PPi exchange by neutral salts is similar to that determined for the leucyl- and valyl-*t*RNA synthetase in *Aesculus* spp.,[26] the lysyl enzyme in *C. ensiformis*,[13] and the glycyl enzyme from rat liver.[27] Bokyo and Fraser[27] suggested that sensitive salt linkages in the enzyme are disrupted at high ionic strength, and that such salt bridges are involved in the conformation of the active centre. The majority of aminoacyl-*t*RNA synthetases, however, are either insensitive to the presence of high salt concentrations, or are slightly stimulated by them.[28]

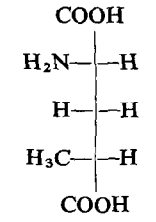
Data obtained from activation studies by the ATP–PPi assay (Table 5) and from inhibition studies on the formation of ^{14}C-glutamyl-*t*RNA (Table 6), indicate that glutamyl-*t*RNA synthetase from *C. bonduc* could utilize only the *threo*-diastereoisomers of the γ-substituted glutamic acids (I and II), i.e. it does not activate its natural product, *erythro*-γ-methyl-L-glutamic acid (IV). The replacement of methyl by hydroxyl at the γ-carbon atom, with retention of configuration, caused little change in the enzyme's affinity for the substrate molecules. Conversely, the enzyme from *H. fulva* only utilized *erythro*-diastereoisomers as substrates (IV and V), showing no affinity for *threo*-γ-hydroxy-L-glutamic acid (II), its own natural product. Again the enzyme was unable to differentiate between substrates bearing either γ-methyl or γ-hydroxyl substitutions of the same stereochemical conformation. The *P. aureus* enzyme had a lower substrate specificity being able to activate both *threo*- and *erythro*-isomers of γ-methyl and γ-hydroxy-L-glutamic acids and, to a lesser extent, the 2(*S*),4(*S*)- and 2(*S*),4(*R*)-isomers of γ-hydroxy-γ-methyl-L-glutamic acid (III and VI, respectively). However this enzyme was probably unable to catalyze the transfer of either isomer of γ-hydroxy-γ-methyl glutamic acid to *t*RNA, because these substituted

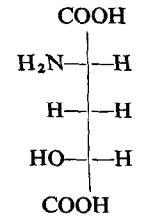
[22] J. E. ALLENDE and C. C. ALLENDE, in *Methods in Enzymology* (edited by K. MOLDAVE and L. GROSSMAN), Vol. XX, p. 210, Academic Press, New York (1971).
[23] A. H. MEHLER and S. K. MITRA, *J. Biol. Chem.* **242**, 5495 (1967).
[24] M. P. DEUTCHER, *Arch. Biochem. Biophys.* **125**, 758 (1968).
[25] F. X. COLE and P. R. SCHIMMEL, *Biochem.* **9**, 3143 (1970).
[26] J. W. ANDERSON and L. FOWDEN, *Biochem. J.* **119**, 691 (1970).
[27] J. BOKYO and M. J. FRASER, *Can. J. Biochem.* **42**, 1677 (1963).
[28] R. B. LOFTFIELD, in *Protein Synthesis* (edited by E. MCCONKEY), Vol. I, Marcel Dekker, New York (1971).

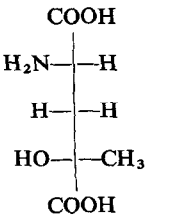

(I) *threo*-γ-Methyl-L-glutamic acid


(II) *threo*-γ-Hydroxy-L-glutamic acid


(III) 2(*S*),4(*S*)-γ-Hydroxy-γ-methyl-L-glutamic acid


(IV) *erythro*-γ-Methyl-L-glutamic acid


(V) *erythro*-γ-Hydroxy-L-glutamic acid


(VI) 2(*S*),4(*R*)-γ-Hydroxy-γ-methyl-L-glutamic acid

derivatives had no action upon the formation of ^{14}C-glutamyl-*t*RNA. A similar difference in substrate specificities in reactions effecting ATP–PPi exchange and ^{14}C-aminoacyl-*t*RNA formation has been noted for other plant[14] and bacterial systems.[29]

It is clear from the results in Table 5 that the two analogue-containing plants, *C. bonduc* and *H. fulva*, have developed discriminatory mechanisms based on altered enzyme specificities that prevents the plants from incorporating their own natural products into protein molecules. Apparently, this has been achieved by specific modifications of the active sites resulting in altered substrate affinities, such that the activation of *erythro*- and *threo*-γ-substituted derivatives, respectively, is prevented. The enzymes from *P. aureus*, which shows less stringent substrate requirements, might be considered an ancestral type, from which the two more specific enzymes have evolved.

EXPERIMENTAL

Plant materials. Seed of *P. aureus* was obtained commercially. Seed of *C. bonduc* was kindly supplied by Dr. A. S. Seneviratne, Dept. of Botany, University of Ceylon, Columbo, 3, Ceylon. *H. fulva* plants were collected from Nuffield Gardens, Regent's Park, London, N.W.1.

Amino acids. threo-γ-Hydroxy-L-glutamic acid was an isolate from *Phormium tenax*.[30] *erythro*-γ-Hydroxy-DL-glutamic acid was donated by Dr. E. E. Dekker (Ann Arbor, Mich., U.S.A.). *erythro*-γ-Methyl-L-glutamic acid was an isolate from *Phyllitis scolopendrium*.[31] *threo*-γ-Methyl-DL-glutamic acid was separated from a synthetic mixture of *erythro*- and *threo*-forms by the method of Blake and Fowden.[31] 2(*S*),4(*S*)-γ-Hydroxy-γ-methyl-L-glutamic acid was also an isolate from *P. scolopendrium*.[31] 2(*S*),4(*R*)-γ-Hydroxy-γ-methyl-L-glutamic acid was a gift from Prof. J. Casimir (Gembloux, Belgium), who isolated the compound from the fungus *Ledenbergia roseoaenea*.[32] γ-Ethylidene-L-glutamic acid was isolated from *Tulipa gesneriana*.[33] γ-Ethyl-L-glutamic acid was obtained by hydrogenation of the γ-ethylidene derivative. *cis*- and *trans*-L-α-(Carboxycyclopropyl)glycines were isolated from *Aesculus parviflora*[34] and *Blighia sapida*,[34] respectively. The γ-ethyl ester of glutamic acid was prepared by the method of Miller and Waelsch.[35]

[29] R. Stern and A. H. Mehler, *Biochem. Z.* **342,** 400 (1965).
[30] P. J. Peterson and L. Fowden, unpublished results.
[31] J. Blake and L. Fowden, *Biochem. J.* **92,** 136 (1964).
[32] J. Jadot, J. Casimir and A. Loffet, *Biochim. Biophys. Acta* **136,** 79 (1967).
[33] L. Fowden, *Biochem. J.* **98,** 57 (1966).
[34] L. Fowden, A. Smith, D. S. Millington and R. C. Sheppard, *Phytochem.* **8,** 437 (1969).
[35] H. K. Miller and H. Waelsch, *Arch. Biochem. Biophys.* **35,** 176 (1952).

γ-Methylene-, β-methyl- and β-hydroxy-glutamic acids were obtained as synthetic racemates. The purity of the analogues was tested by PC using *n*-BuOH–HOAc–H_2O (90:10:29). Only samples showing no glutamate contamination were tested as enzyme substrates.

Radioisotopic chemicals. L-[U-^{14}C] Glutamic acid (225 μCi/μmol) 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 μCi/μmol.

Enzyme fractionation. All operations were carried out in a cold room at 1–4°. (a) *P. aureus* seed meal was ground manually with extraction medium (0·1 M Tris-HCl buffer, pH 7·5, containing 20 mM $MgCl_2$ and 10% (w/v) glycerol) (4 ml/g seed). The macerate was squeezed through muslin and centrifuged at 12 000 *g* for 15 min. $(NH_4)_2SO_4$ was added to give 45% saturation; protein sedimented at 12 000 *g* for 15 min was discarded and further $(NH_4)_2SO_4$ added to the supernatant to give 55% saturation. The protein precipitated was collected by centrifuging at 12 000 *g* for 15 min, and redissolved in extraction buffer (5 ml). The solution was passed through a column of Sephadex G-25 (equilibrated with extraction buffer) of bed volume 80 ml and the 35–50 ml effluent fraction collected. Acetone at —10° was slowly added to the eluate to produce a concentration of 60% (v/v): the mixture was centrifuged at 10 000 *g* for 10 min and the pellet discarded. Further acetone was added to give a final concentration of 80% (v/v), and the precipitate was collected by centrifugation at 10 000 *g* for 10 min. The pellet was redissolved in 0·05 M Tris-HCl buffer, pH 7·5, containing 20 mM $MgCl_2$ and 10% (w/v) glycerol. Further purification was achieved by chromatography on a DEAE-cellulose column (30 × 1·5 cm dia.) equilibrated with the same medium, and developed with a linear gradient of 0–0·2 M KCl; fractions (3 ml) were collected, and alternate tubes assayed for enzyme activity. (b) *C. bonduc* seeds (minus testas) were extracted with 6 ml extraction medium/g dry seed. A purification procedure essentially similar to that employed for *P. aureus* was followed. (c) *H. fulva* leaves were macerated with extraction buffer (20 mM potassium thioglycollate added), 1 ml/g fresh tissue, in a M.S.E. Atomix blender. A slight alteration was made in $(NH_4)_2SO_4$ fractionation procedure, whereby a 40–50% saturated fraction was obtained. After treatment on Sephadex G-25, a 75–90% (v/v) acetone fraction was prepared and used as a source of glutamyl-*t*RNA synthetase.

tRNA isolation. The method of Vanderhoef *et al.*,[36] was employed to extract *t*RNA from *P. aureus* and *C. bonduc* seed meal. Double the suggested volume of Tris-HCl buffer was utilized in the initial extraction method. An A_{260}/A_{280} ratio of 2·1 and an A_{260}/A_{290} ratio of 3·5 compared favourably with values of 1·9 and 3·19 obtained by Vanderhoef *et al.*[36] A yield of 5–7 mg/100 g seed was obtained for both species.

Protein determination. The method of Lowry *et al.*[37] was normally adopted, using bovine serum albumin as a standard. Fractions eluted from DEAE-cellulose columns were subject to interference by KCl, and then protein contents were estimated from extinction valves measured at 260 and 280 nm by the method of Layne.[38]

Assay procedures. (a) *ATP–PPi exchange system.* The method was based on that described by DeMoss and Novelli,[39] and by Smith and Fowden.[14] The incubation mixture normally contained: Tris-HCl buffer (100 μmol) pH 7·8, $MgCl_2$ (10 μmol), ATP (4 μmol), [^{32}P] pyrophosphate (4 μmol), 0·5 μCi), glutamic acid (35 μmol) and enzyme preparation: total volume, 1 ml. Analogues, when tested in competition with glutamate, were added at 70 μmol/ml. When other aminoacyl-*t*RNA synthetases were assayed individually, amino acid concentrations of 5 mM were used. The exchange rates were calculated by the method of Davie *et al.*[40] (b) *^{14}C-Glutamyl-tRNA formation.* The method followed was that of Mans and Novelli.[41] The incubation mixture contained: $MgCl_2$ (5 μmol), ATP (5 μmol), ^{14}C-glutamic acid ($4·4 \times 10^{-3}$ μmol, 1·0 μCi), Tris-HCl buffer, pH 8·0 (10 μmol), *t*RNA preparation (0·25 mg) and enzyme preparation; total vol. 1 ml. Analogues, when tested for their inhibitory effect, were added at 1·76 μmol/ml concentrations. The reaction was allowed to proceed for 5 min at 37°. Radioactivity was determined by counting in a Tri-Carb liquid scintillation counter.

[36] L. N. VANDERHOEF, R. F. BOHANNON and J. L. KEY, *Phytochem.* **9**, 2291 (1970).
[37] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[38] E. LAYNE, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 3, p. 447, Academic Press, New York (1957).
[39] J. A. DEMOSS and G. D. NOVELLI, *Biochim. Biophys. Acta* **22**, 49 (1956).
[40] E. W. DAVIE, V. V. KONINGSBERGER and F. LIPMANN, *Arch. Biochem. Biophys.* **65**, 21 (1956).
[41] R. J. MANS and G. D. NOVELLI, *Arch. Biochem. Biophys.* **94**, 48 (1961).