Properties and Substrate Specificity of the Leucyl-, the Threonyl- and the Valyl-Transfer-Ribonucleic Acid Synthetases from *Aesculus* Species

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(Received 18 June 1970)

1. Leucyl- and threonyl-tRNA synthetases were partially purified up to 100-fold and 30-fold respectively from cotyledons of *Aesculus hippocastanum* and were largely separated from the other aminoacyl-tRNA synthetases. Valyl-tRNA synthetase was purified 25-fold from cotyledons of *Aesculus californica*. 2. Some properties are reported for the three enzymes when assayed by the [³²P]pyrophosphate-ATP exchange technique. 3. β -(Methylenecyclopropyl)alanine, isoleucine, azaleucine, norleucine and γ -hydroxynorvaline acted as alternative substrates for the leucyl-tRNA synthetase; the enzyme's affinity for β -(methylenecyclopropyl)-alanine and for isoleucine was about 80-fold less than that exhibited for leucine. 4. α -Cyclopropylglycine and α -cyclobutylglycine acted as alternative substrates for the valyl-tRNA synthetase.

The cotyledons of *Aesculus* seeds are rich sources of phenylalanyl-tRNA synthetase. During purification of this enzyme from seeds of various species of *Aesculus*, several fractions were obtained that exhibited high amino acid-dependent pyrophosphate exchange activity although no activity was demonstrated when phenylalanine was used as substrate (Anderson & Fowden, 1970). These fractions were surveyed for individual aminoacyl-tRNA synthetases and some were found to contain only one major aminoacyl-tRNA synthetase, purified approx. 30–100-fold. For example, the leucyl- and threonyl-tRNA synthetase from *A. hippocastanum* and the valyl-tRNA synthetase from *A. californica* were present in discrete fractions obtained during the preparation of the phenylalanyl-tRNA synthetase from the respective species, and so these fractions were used as sources of the respective enzymes without further purification. The kinetic properties of these three enzymes are described; particular attention was given to the possibility that amino acids, structurally related to leucine, threonine or valine (some of which are plant products), might serve as substrates for the respective enzymes.

METHODS

Chemicals. The L-isomers of leucine, isoleucine, valine and threonine were obtained from Mann Research Laboratories Inc., New York, N.Y., U.S.A. The naturally

occurring isomers of the following amino acids were obtained by isolation; homoisoleucine (*A. californica*; Fowden & Smith, 1968); γ -hydroxynorvaline (*Lathyrus odoratus*; Fowden, 1966); 2-amino-4-hydroxymethylhex-5-ynoic acid (*Euphoria longan*; Sung, Fowden, Millington & Sheppard, 1969); α -(methylenecyclopropyl)glycine (*Litchi chinensis*; Gray & Fowden, 1962); hypoglycin A [β -(methylenecyclopropyl)alanine] [*Blighia sapida*; Ellington, Hassall, Plimmer & Seaforth, 1959; obtained after hydrolysis of the γ -glutamyl peptide (i.e. hypoglycin B) to ensure absence of leucine]. The following compounds were synthesized chemically and were racemates: α -cyclopropylglycine, α -cyclobutylglycine and ethallylglycine (from Professor W. Shive, Austin, Tex., U.S.A.), azaleucine and norleucine (from Calbiochem Ltd., London W.1., U.K.), 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid 'a' and 'b' isomers (Dr H. N. Christensen and Dr H. S. Tager, Ann Arbor, Mich., U.S.A.), and 1-amino-2-nitrocyclopentane-1-carboxylic acid (Dr D. Broadbent, Imperial Chemical Industries Ltd., Wilmslow, Cheshire, U.K.). 1-Aminocyclopentane-1-carboxylic acid was a gift from Dr L. Berlinguet (Quebec, Que., Canada).

The sources of other reagents used in enzyme assays were the same as those described by Anderson & Fowden, (1970).

Extraction and purification of the leucyl- and the threonyl-tRNA synthetases from cotyledons of *A. hippocastanum* seeds. The method was identical with that used for the extraction and purification of phenylalanyl-tRNA synthetase from *A. hippocastanum*. The leucyl- and the threonyl-tRNA synthetases were separated from the phenylalanyl-tRNA synthetase during chromatography on DEAE-cellulose at pH 7.5 (Anderson & Fowden, 1970).

Extraction and purification of the valyl-tRNA synthetase from cotyledons of *A. californica* seeds. This enzyme was extracted from seeds harvested in 1967 and was purified

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as described for the phenylalanyl-tRNA synthetase from *A. californica* (Anderson & Fowden, 1970) except that the material precipitated between 35 and 45% (v/v) acetone was used as the source of enzyme.

All other procedures were as described by Anderson & Fowden (1970) except that KCl was omitted from the enzyme assays of the valyl-tRNA synthetase and the leucyl-tRNA synthetase as these enzymes are strongly inhibited by KCl.

RESULTS

Purification of the leucyl- and the threonyl-tRNA synthetases from A. hippocastanum. Leucyl-tRNA synthetase was present in peak II from the DEAE-cellulose column described by Anderson & Fowden (1970). It was consistently eluted after phenylalanyl-tRNA synthetase; threonyl-tRNA synthetase (contained in peak I) was eluted before the phenylalanyl-tRNA synthetase. The aminoacyl-tRNA synthetases present in peaks I and II are shown in Table 1. The threonyl-tRNA synthetase was virtually uncontaminated by the leucyl- and the phenylalanyl-tRNA synthetases, and the activity of the most active contaminant (valyl-tRNA synthetase) was only 15% of that of the threonyl-tRNA synthetase. The specific activity of threonyl-tRNA synthetase in peak I (97 units/mg of protein) represents a 30-fold purification relative to the activity in crude extracts. Protein from peak I exhibited no endogenous pyrophosphate exchange activity, indicating the absence of endogenous substrates.

The leucyl-tRNA synthetase (peak II) contained virtually no threonyl-tRNA synthetase, but was never fully separated from the phenylalanyl-

tRNA synthetase. Nevertheless, the pyrophosphate exchange activity with phenylalanine as substrate never exceeded 17% of the value obtained with leucine. The high exchange activity measured in the presence of isoleucine was ascribed to the ability of isoleucine to act as an alternative substrate for the leucyl-tRNA synthetase. Several lines of evidence (discussed below) suggest that there is no isoleucyl-tRNA synthetase in peak II. Protein from peak II exhibited no endogenous pyrophosphate exchange activity. The specific activity of the leucyl-tRNA synthetase in peak II was 220 and 1190 units/mg of protein for seeds harvested in 1968 and 1969 respectively; these represent purifications of 25- and 100-fold compared with the activity present in crude extracts.

Purification of the valyl-tRNA synthetase from A. californica. The 35–45%-acetone fraction prepared from seeds of the 1967 and 1969 harvests contained a valyl-tRNA synthetase that was purified approx. 25-fold compared with the activity of crude extracts. The activity of the most active contaminating synthetase (leucyl-tRNA synthetase) from seeds harvested in 1967 was less than 20% of the activity of the valyl-tRNA synthetase (Table 2). The leucyl-tRNA synthetase can be easily removed by chromatography on DEAE-cellulose at pH 7.5 (Anderson & Fowden, 1970). The 35–45%-acetone fraction prepared from seeds harvested in 1969 provided a good source of valyl-tRNA synthetase, but contamination by the leucyl-, the threonyl- and the phenylalanyl-tRNA synthetase was high.

Seeds of *A. californica* harvested in 1968 contained very little valyl-tRNA synthetase, but the phenylalanyl-tRNA synthetase was very active

Table 1. *Survey of the aminoacyl-tRNA synthetase activities in peak I (threonyl-tRNA synthetase) and peak II (leucyl-tRNA synthetase) from the DEAE-cellulose column during chromatography of phenylalanyl-tRNA synthetase from A. hippocastanum*

The specific activity of threonyl-tRNA synthetase in peak I was 97 units/mg of protein and the specific activity of leucyl-tRNA synthetase in peak II was 221 units/mg of protein. Synthetases absent from the material added to the DEAE-cellulose column (see Anderson & Fowden, 1970) were not assayed.

Substrate	Peak I	Peak II
	Substrate-dependent pyrophosphate exchange relative to threonine (%)	Substrate-dependent pyrophosphate exchange relative to leucine (%)
Glycine	0	1.2
Isoleucine	0	55*
Leucine	0.6	100
Methionine	0	0
Phenylalanine	2.4	12
Serine	5.2	9.6
Threonine	100	3.2
Tryptophan	0	0
Valine	14.6	6.0

* The high value for isoleucine in peak II represents activation of isoleucine by leucyl-tRNA synthetase and not the presence of an isoleucyl-tRNA synthetase (see the text).

Table 2. *Survey of the aminoacyl-tRNA synthetases in the 35–45% acetone fraction prepared from seeds of A. californica harvested in 1967*

The activities of the aminoacyl-tRNA synthetases not listed were less than 1% of the activity of valyl-tRNA synthetase. The specific activity of valyl-tRNA synthetase was 40.5 units/mg of protein.

Substrate	Substrate-dependent pyrophosphate exchange relative to valine (%)
Alanine	2.0
Aspartic acid	14.8
Glycine	9.6
Isoleucine	12.3
Leucine	19.4
Threonine	2.4
Valine	100

Fig. 1. Inhibitory effect on leucyl-tRNA synthetase activity produced by adding KCl to the standard assay mixture. Details of the assay are given in the Methods section.

(Anderson & Fowden, 1970). The 1968 crop was harvested 4 weeks earlier in the growing season than were seeds collected in 1967, and this might explain the different activities of these two enzymes in the two batches of seed. Variation in activity of the aminoacyl-tRNA synthetases with physiological development are well documented (Anderson & Fowden, 1969; Anderson & Cherry, 1969).

Properties of the leucyl-tRNA synthetase from A. hippocastanum. The affinity of this synthetase for leucine was extremely high (K_m 9 μ M); a concentration of 0.1 mM-L-leucine was almost sufficient to saturate the enzyme ($v = 92\%$ V). Leucyl-tRNA synthetase was strongly inhibited by high concentrations of potassium chloride (Fig. 1). Other

Table 3. *Inhibition of leucyl-tRNA synthetase from A. hippocastanum by neutral salts present in the normal assay mixture*

Salt	Inhibition (%)
NaCl (0.67 M)	77
NH ₄ Cl (0.67 M)	87
KNO ₃ (0.67 M)	92
KBr (0.67 M)	87
LiCl (0.67 M)	88
CaCl ₂ (0.33 M)	99
MnCl ₂ (0.33 M)	99
CsCl (0.33 M)	98

Table 4. *Alternative substrates of the leucyl-tRNA synthetase from A. hippocastanum*

Activities are expressed as percentages of V for leucine as substrate. The pyrophosphate exchange (relative to V for leucine) of the most active contaminating synthetase (phenylalanyl-tRNA synthetase) was 12%. Amino acid concentrations are given in parentheses and refer to the L-isomer.

Substrate	Pyrophosphate exchange (% of V for leucine)
L-Hypoglycin A (5 mM)	60
DL-Azaleucine (10 mM)	20
DL-Norleucine (10 mM)	10
L- γ -Hydroxynorvaline (10 mM)	17
L-Isoleucine (4 mM)	75

Table 5. *Kinetics of the activation of leucine, isoleucine and hypoglycin A by the leucyl-tRNA synthetase from A. hippocastanum*

Substrate	$K_m \pm$ S.E.M. (mM)	$V \pm$ S.E.M. (% of V for leucine)
L-Leucine	0.009 ± 0.001	100 ± 3
L-Isoleucine	0.71 ± 0.11	78 ± 3
L-Hypoglycin A	0.78 ± 0.05	55 ± 3

neutral salts also inhibited the enzyme (Table 3). This suggests that high ionic strength may slightly modify the configuration around the active centre of the enzyme. Caesium chloride and salts yielding bivalent cations caused almost total inhibition; this could be due to the cumulative effects of high ionic strength, interference with Mg^{2+} action, and precipitation of pyrophosphate by the bivalent cations.

Leucyl-tRNA synthetase from *A. hippocastanum* activated several amino acids structurally related to leucine (Table 4). All the substrates listed in Table 4 (with the exception of γ -hydroxynorvaline)

caused a decrease in leucine-dependent pyrophosphate exchange activity when incubated in mixtures containing L-leucine. It is most unlikely that γ -hydroxynorvaline is activated by a contaminating synthetase, because the activity of the most active contaminant (phenylalanyl-tRNA synthetase) was 12% of that of leucyl-tRNA synthetase.

Evidence that isoleucine was activated by the leucyl-tRNA synthetase from *A. hippocastanum* is as follows. (i) The affinity of peak II protein for isoleucine was approx. 80-fold less than for leucine (Table 5). Alternative substrates for other aminoacyl-tRNA synthetases generally show affinities that are at least 30-fold less than those measured for the normal substrate (Peterson, 1967; Smith & Fowden, 1968; Anderson & Fowden, 1970). (ii) Extremely high concentrations of isoleucine were required to saturate the isoleucine-dependent pyrophosphate exchange activity of peak II protein, but the exchange rate at saturation was still less than that obtained with leucine (Table 5): this eliminates the possibility that exchange was due to leucine contamination of the isoleucine sample. (iii) By using selected concentrations of leucine and isoleucine, competition between these two substrates for the dominant aminoacyl-tRNA synthetase in peak II protein could be demonstrated

(Table 6). The high concentration of isoleucine, relative to leucine, required to demonstrate competition suggests that isoleucine was an alternative substrate for the leucyl-tRNA synthetase. (iv) Isoleucine-dependent pyrophosphate exchange activity of peak II protein showed a similar sensitivity to potassium chloride as did the leucine-dependent exchange activity; 0.67 M-potassium chloride caused 93% inhibition of isoleucine-dependent activity.

Activation of hypoglycin A as an alternative substrate was examined in detail since this compound forms the toxic principle of immature seeds of *Blighia sapida* (Ellington *et al.* 1959). Kinetic data relating to the activation of hypoglycin A by the leucyl-tRNA synthetase from *A. hippocastanum* are given in Table 5 and the conditions for demonstrating competition between leucine and hypoglycin A are described in Table 6. Since *V* for hypoglycin A was only 55% of the value determined for leucine, this eliminated the possibility that the pyrophosphate exchange activity stimulated by hypoglycin A was due to contamination by leucine. Hypoglycin A was not activated by phenylalanyl-tRNA synthetase (Anderson & Fowden, 1970).

The following compounds were tested as substrates for leucyl-tRNA synthetase and were found to be inactive and to have no effect on the rate of pyrophosphate exchange determined in the presence of leucine: valine, homoisoleucine, ethylallyl glycine, 2-amino-4-methylhex-4-enoic acid, 2-amino-4-hydroxymethylhex-5-ynoic acid, α -(methylenecyclopropyl)glycine, 1-aminocyclopentane-1-carboxylic acid and 1-amino-2-nitrocyclopentane-1-carboxylic acid. The 'a' and 'b' isomers of 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (Christensen, Handlogten, Lam, Tager & Zand, 1969) were not activated, but the compounds exhibited competition with leucine; the 'a' and 'b' isomers, when present at 10 mM concentration, decreased the leucine-dependent pyrophosphate exchange activity by 65% and 55% respectively with 0.1 mM-leucine as substrate.

Properties of the valyl-tRNA synthetase from A. californica. The K_m of this enzyme with valine as substrate was 120 μ M. Valyl-tRNA synthetase

Table 6. *Experimental conditions for demonstrating competition between isoleucine and leucine and between hypoglycin A and leucine for the leucyl-tRNA synthetase from A. hippocastanum*

Substrate concentrations and standard deviations are shown in parentheses. The S.E.M. calculated from an analysis of variance was $\pm 4.8\%$.

Substrate(s)	<i>V</i> (% of <i>V</i> with 0.1 mM-leucine as substrate)
L-Leucine (0.1 mM)	100
L-Isoleucine (1.0 mM)	42
L-Leucine (0.1 mM)	84
+ L-isoleucine (1.0 mM)	
L-Hypoglycin A (10 mM)	52
L-Leucine (0.1 mM)	80
+ L-hypoglycin A (10 mM)	

Table 7. *Alternative substrates for the valyl-tRNA synthetase from A. californica, and conditions for demonstrating competition of these substrates with valine*

Amino acid concentrations are given in parentheses and refer to the L-isomer.

Substrate	Pyrophosphate exchange (% of <i>V</i> for valine)	Conditions for demonstrating competition of substrate with valine	
		Substrate (mM)	Valine (mM)
DL-Cyclopropylglycine (2.9 mM)	12.8	2.9	1
DL-Cyclobutylglycine (10 mM)	8.3	10	2

was extremely sensitive to potassium chloride; enzyme activity was inhibited 93% by 0.5M-potassium chloride.

Valyl-tRNA synthetase (prepared from seed harvested in 1969) activated several amino acids in addition to valine (Table 7). The enzyme was contaminated by the threonyl-, the leucyl- and the phenylalanyl-tRNA synthetases, so it was essential to demonstrate competition between alternative substrates and valine (Anderson & Fowden, 1970): the conditions used are shown in Table 7. Although the 'b' isomer of 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (at 10mM concentration) was not activated by this enzyme, it decreased the valine-dependent pyrophosphate exchange activity by 20% when 2mM-valine was used as substrate.

Properties of the threonyl-tRNA synthetase from A. hippocastanum. The K_m of this enzyme for threonine substrate was 100 μ M. Enzyme activity was almost unaffected by 0.5M-potassium chloride. Homoserine was not activated and did not decrease the threonine-stimulated pyrophosphate exchange activity.

DISCUSSION

The most important feature of the purification of specific aminoacyl-tRNA synthetases for studies of their amino acid substrate specificity is the removal of contaminating synthetases (Anderson & Fowden, 1970). The leucyl-, the threonyl- and the valyl-tRNA synthetases described in this paper were largely separated from each other and from the other aminoacyl-tRNA synthetases (Tables 1 and 2). The least pure enzyme was leucyl-tRNA synthetase, which contained appreciable phenylalanyl-tRNA synthetase; however, the activity of this contaminating enzyme never exceeded 17% of that of the leucyl-tRNA synthetase and in most experiments the contamination was considerably less. Residual endogenous pyrophosphate exchange activity is another criterion for assessing the degree of purification of aminoacyl-tRNA synthetases; the endogenous pyrophosphate exchange activity measured with any of the three enzymes never exceeded 3% of the exchange occurring when the appropriate amino acid substrate was used. Purification of the threonyl- and the valyl-tRNA synthetases, in terms of increased specific activity, was only moderately successful (25–30-fold for each enzyme), but a more satisfactory purification (100-fold) was achieved for the leucyl-tRNA synthetase: the three synthetases arose in practice as by-products of the purification of phenylalanyl-tRNA synthetase and not as the result of a specially designed fractionation procedure.

The leucyl-tRNA synthetase from *A. hippocastanum* has a high affinity for leucine; the K_m value is approx. 25-fold less than that determined

for the leucyl-tRNA synthetase purified from *Lupinus luteus* by similar procedures (Legocki & Pawelkiewicz, 1967). The K_m for leucine determined for the leucyl-tRNA synthetase from *A. hippocastanum* is lower than any other comparable K_m value recorded for amino acid-aminoacyl-tRNA synthetase combinations from plant sources (Attwood & Cocking, 1965; Legocki & Pawelkiewicz, 1967; Fowden & Frankton, 1968; Smith & Fowden, 1968; Anderson & Fowden, 1970) and indicates enzyme-substrate affinities similar to those calculated for synthetases from *Escherichia coli* (Peterson, 1967). The affinities of the threonyl- and the valyl-tRNA synthetases for their respective substrates are consistent with values recorded for most aminoacyl-tRNA synthetases from plant tissues.

The leucyl- and the valyl-tRNA synthetases are extremely sensitive to the ionic composition of the assay medium (Fig. 1 and Table 3) and in this respect differ from the phenylalanyl- and the threonyl-tRNA synthetases, which are generally insensitive to 0.67M-potassium chloride: potassium chloride sometimes produced a slight increase in the activity of phenylalanyl-tRNA synthetase (Anderson & Fowden, 1970). Boyko & Fraser (1964) reported that the glycyl-tRNA synthetase of rat liver was inhibited by K^+ and NH_4^+ , and they suggested that the glycyl-tRNA synthetase contains salt-sensitive bridges that are disrupted at high ionic strength. The salt-sensitivity of the leucyl- and the valyl-tRNA synthetases is consistent with this idea.

Several aminoacyl-tRNA synthetases activate more than one protein amino acid. Isoleucyl-tRNA synthetase from *E. coli* activates valine in addition to isoleucine, although the enzyme's affinity for valine is some 100-fold less than that of for isoleucine, and V (valine) is only 50% of I (isoleucine) (Bergman, Berg & Dieckmann, 1961; Loftfield & Eigner, 1961). Similarly the valyl-tRNA synthetase from *E. coli* activates threonine in addition to valine, but again the affinity for threonine is about 100-fold less than that for valine (Bergman *et al.* 1961). The activation of isoleucine by leucyl-tRNA synthetase (Tables 5 and 6) provides a further example of synthetases activating more than one protein amino acid. The affinity of the leucyl-tRNA synthetase for isoleucine is about 80-fold less than for its normal substrate, so the amount of isoleucyladenylate synthesized *in vivo* must be small in relation to the synthesis of leucyladenylate. It remains to be seen whether tRNA^{Leu} will accept isoleucyl residues from isoleucyladenylate in the presence of leucyl-tRNA synthetase. Legocki & Pawelkiewicz (1967) observed a decrease in the activation of leucine in the presence of methionine and isoleucine and suggested that this

could be caused either by competition between leucine and isoleucine (or methionine), or by allosteric transition of the enzyme. In our studies with leucyl-tRNA synthetase, the possibility of allostery is ruled out by the demonstration of classical Michaelis-Menten kinetics for the activation of both leucine and isoleucine.

Anderson & Cherry (1969) presented evidence for the existence of at least six species of tRNA^{Leu} and their data imply the existence of at least two different leucyl-tRNA synthetases in the tissues of *Glycine max*. In similar studies Williams (1969) has observed four species of tRNA^{Leu} and two leucyl-tRNA synthetases in extracts of leaves of *Phaseolus vulgaris*: each of the two enzymes has a clear and absolute specificity among the four tRNA^{Leu} acceptors. In our experiments chromatography on DEAE-cellulose provided no evidence for the existence of more than one enzyme in *A. hippocastanum*. However, no attempt was made to measure leucyl-tRNA synthetase in fractions before chromatography on DEAE-cellulose, because the purification scheme that led to the recovery of leucyl-tRNA synthetase was designed for the purification of phenylalanyl-tRNA synthetase. The possibility that isoenzymes of leucyl-tRNA synthetase had been discarded before chromatography cannot be discounted. The same is true of the threonyl- and the valyl-tRNA synthetases.

Possibly for similar reasons the fractionation procedure used did not reveal the presence of a specific isoleucyl-tRNA synthetase. The literature describing aminoacyl-tRNA synthetases contains little information relating to the purification of specific isoleucyl-tRNA synthetases, except for enzymes of bacterial origin. Certainly no purification of this enzyme from a higher-plant source seems to have been attempted; in those cases where activation rates were reported for isoleucine and leucine by mixtures of aminoacyl-tRNA synthetases present in higher plants (Moustafa & Proctor, 1962; Smith & Fowden, 1968; Anderson & Rowan, 1966; Anderson & Fowden, 1969; Scott & Morris, 1969) the rates measured for isoleucine relative to leucine (i.e. 55–80% of the leucine value) were of the same order as those recorded in Tables 1, 4 and 5 with the leucyl-tRNA synthetase from *A. hippocastanum* and saturating concentrations of isoleucine. Obviously a precise identification of a specific isoleucyl-tRNA synthetase in plant extracts is now required.

The other compounds that, like isoleucine, could act as alternative substrates for the leucyl-tRNA synthetase of *A. hippocastanum* (Table 4) can be represented by the general structure

i.e. the carbon skeleton is not branched before the

γ -carbon atom, if at all. For analogue molecules accepted by the enzyme, R can be represented as:

γ -Hydroxynorvaline

Hypoglycin A

Azaleucine

Norleucine

Except in the instance of isoleucine, compounds having a carbon skeleton branched at the β -carbon atom, e.g. valine and α -(methylenecyclopropyl)-glycine, were completely inactive as substrates for the leucyl-tRNA synthetase. Perhaps the most surprising observation is the relatively high exchange rate measured for hypoglycin A (Table 4). This substance, which has a molecule larger than the normal substrate, nevertheless has all four carbon atoms forming R arranged in a coplanar manner, in contrast with homoisoleucine [$R = -CH(CH_3) \cdot CH_2 \cdot CH_3$], which exhibited no substrate activity. The activation of azaleucine was not unexpected in view of the striking growth inhibition noted when this analogue was added to exponentially growing cultures of *E. coli* and *Leuconostoc dextranicum* (Smith, Bayliss & McCord, 1965). Likewise, the substitution of a hydroxyl group for the methyl group (of leucine) in γ -hydroxynorvaline is a type of modification known previously to be compatible with analogue behaviour (Richmond, 1962). A somewhat comparable situation exists in which β -hydroxynorvaline acts as an antagonist of isoleucine (and threonine and methionine) in *E. coli* strain K12 (Cohen, Patte, Truffa-Bachi, Sawas & Doudoroff, 1965).

The role of leucine analogue now demonstrated for hypoglycin A raises the question whether the toxicity of this natural product of *B. sapida* is fully explained in terms of its hypoglycaemic effect (see review by Bressler, Corredor & Brendel, 1969). A study of the substrate specificity of mammalian leucyl-tRNA synthetase would be revealing at the present time.

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