The Aminoacylation of Transfer Ribonucleic Acid

INHIBITORY EFFECTS OF SOME AMINO ACID ANALOGUES WITH ALTERED SIDE CHAINS

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Several amino acid analogues that are able to replace amino acid residues in binding positions of the biologically active C-terminal tetrapeptide amide sequence, Trp-Met-Asp-PheNH₂, of the gastrins were examined for their ability to inhibit the aminoacylation of tRNA in an *Escherichia coli* and rat liver system. Although in both systems the amino acid side chains are involved in the recognition process, the structural requirements of the side chain in the two systems are not comparable. Analogues of methionine and phenylalanine behaved similarly in the *E. coli* and rat liver systems, whereas analogues of tryptophan behaved differently. From the results it is possible to suggest structural features of the amino acid side chains which are required for recognition by the aminoacyl-tRNA synthetases.

In the last decade or so it has been shown that amino acid analogues are able to replace amino acids in enzymes and peptide hormones while the biological integrity of the molecule is maintained. One of the peptide hormones in which the effect of amino acid replacement by analogues on biological activity has been studied extensively is gastrin. Here the C-terminal tetrapeptide amide Trp-Met-Asp-PheNH₂ contains all the biological activity of the whole molecule (Tracy & Gregory, 1964). Morley and his co-workers (Morley, 1968, 1969) have shown that the tryptophan, methionine and phenylalanine residues can be replaced by a considerable number of amino acid analogues having altered side chains, but very little change can be made at the aspartic acid position if biological activity is to be retained. From these results it has been proposed that the tryptophan, methionine and phenylalanine residues are involved in 'binding' whereas the aspartic acid has a functional role (Morley, 1968).

The mechanism of recognition between amino acids and their specific aminoacyl-tRNA synthetase, and between the aminoacyl-AMP-synthetase complex and tRNA, is not yet understood. Since the covalent bonds between the amino acid and the AMP-synthetase complex, and between the amino acid and its respective tRNA, are of the same nature for all amino acids, the recognition between the amino acid and its specific synthetase and possibly its specific tRNA must depend largely on the structure of the

amino acid side chain. Similarly it has been proposed that it is the shape of the amino acid side chain that is important in the 'binding' positions of the gastrin C-terminal tetrapeptide. These side chains must fit the specific gastrin receptor site (Morley, 1968).

It is known that certain amino acid analogues can be incorporated into proteins (Richmond, 1962), and some of these analogues have been examined for their ability either to participate in the aminoacyl-tRNA synthetase-catalysed ATP/PP_i exchange reaction, or to aminoacylate tRNA.

In the present study we have sought to determine the effect of amino acid analogues, which can successfully replace 'binding' residues in the gastrin tetrapeptide, on the aminoacylation of tRNA from *Escherichia coli* and rat liver. The object of the study has been twofold: (1) to determine whether the side-chain features involved in recognition of the gastrin binding sites are in any way similar to those required for recognition of the aminoacyl-tRNA synthetase; (2) to contribute to our understanding of the mechanism of the recognition between amino acids and their cognate synthetases. The present paper surveys our initial findings.

The recognition between the analogues and the aminoacyl-tRNA synthetases has been determined by measurement of the extent of inhibition of aminoacyl-tRNA formation with the natural amino acid by the analogue.

From the present results it has been possible to draw some conclusion as to the features in the amino acid side chain that are recognized by the synthetase.

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Materials and Methods

Chemicals

Reagent-grade chemicals were used. *S*-Ethyl-L-cysteine and L-hexahydrophenylalanine were a gift from Dr. J. S. Morley, Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire, U.K. DL-Homocysteine was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. All other non-labelled amino acid analogues were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. L-[14 C]Aspartic acid (229 Ci/mol), L-[14 C]isoleucine (342 Ci/mol), L-[14 C]leucine (331 Ci/mol), L-[14 C]-methionine (54 Ci/mol), L-[14 C]phenylalanine (513 Ci/mol), L-[14 C]threonine (228 Ci/mol), L-[14 C]tryptophan (52 Ci/mol) and L-[14 C]tyrosine (468 Ci/mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. 14 C-labelled amino acids whose specific radioactivities were above 100 μ Ci/mol were diluted with unlabelled L-amino acid to adjust their specific radioactivity to 100 μ Ci/mol. All 14 C-labelled amino acids were diluted with water to a final concentration of 10 μ Ci/ml. Scintillation fluid was composed of 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene (Packard Instruments Ltd., Caversham, Berks., U.K.) in 1 litre of toluene.

tRNA and aminoacyl-tRNA synthetases

Unfractionated tRNA prepared from *Escherichia coli* by the method of Zubay (1962) was a generous gift from Dr. S. Nishimura, National Cancer Centre Research Institute, Tokyo, Japan.

Rat liver tRNA was prepared by the method of Delhas & Staehelin (1966). The livers were obtained from white rats bred in this Department and which originated from Carworth Europe CFHB strain.

A mixture of *E. coli* aminoacyl-tRNA synthetases was prepared by a method similar to that described by Nirenburg (1963). Frozen *E. coli* cells (20 g of strain B, purchased from Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) and 20 g of cold alumina (Sigma type 305) were ground for 15 min in a pre-cooled mortar at 4°C, the temperature at which all subsequent procedures were performed. The resulting paste was suspended in 35 ml of standard KCl buffer (0.01 M-Tris/HCl, pH 7.5, 0.06 M-KCl, 0.01 M-magnesium acetate and 6 mM-2-mercaptoethanol) and the cell debris and alumina were removed from the suspension by centrifugation for 20 min at 20000g (at $r_{\max}$ in a Sorvall SS34 rotor). The supernatant was centrifuged for 30 min at 30000g (at $r_{\max}$ in a Sorvall SS34 rotor), and the resultant supernatant was centrifuged for 3 h at 100000g (at $r_{\max}$ in a Beckman 42.1 rotor). The upper two-thirds of the 100000g supernatant was carefully decanted and dialysed against

1.5 litres of buffer for 18 h, with three changes of buffer. The pH of the diffusate was adjusted from pH 6.8 to pH 7.0 with 0.1 M-Tris solution. A volume of streptomycin sulphate solution, equal to one-tenth of the supernatant volume, was slowly added to the supernatant and the mixture was left stirring for 3 h. The precipitate was removed by centrifugation for 20 min at 20000g (at $r_{\max}$ in a Sorvall SS34 rotor). Protein was precipitated from the supernatant by the slow addition of solid $(\text{NH}_4)_2\text{SO}_4$. The material that was precipitated between a saturation of 40 and 65% $(\text{NH}_4)_2\text{SO}_4$, and that was found to be most enriched in aminoacyl-tRNA synthetase activity, was collected. This was dissolved in a minimal volume of standard KCl buffer and dialysed against 1.5 litres of the same buffer, with three changes over 18 h. The protein concentration was measured with Folin-Ciocalteu reagent (Lowry *et al.*, 1951), and the nucleic acid content of each fraction by the u.v.-ratio method (Layne, 1957). The crude synthetase solution was frozen by adding dropwise to a beaker containing liquid N_2 and the frozen pellets were stored in liquid N_2 .

A mixture of rat liver aminoacyl-tRNA synthetases was prepared as described by Nishimura & Weinstein (1969). The enzyme solution was frozen and stored by the same method as that described for the *E. coli* preparation.

Assay of amino acid-acceptor activity of tRNA

The aminoacylation of tRNA was assayed by a modification of the method described by Hoskinson & Khorana (1965). Optimal conditions for aminoacylation were as follows. The standard reaction mixture consisted of 25 mM-Tris/HCl (pH 7.5), 5 mM-MgCl₂, 0.5 mM-EDTA and 25 mM-ATP, and contained 0.05–0.2 mg of tRNA, approx. 0.06 mg of synthetase preparation from *E. coli* or 0.19 mg of synthetase preparation from rat liver, and 0.05 μ Ci of a 14 C-labelled amino acid in a total volume of 250 μ l.

The reaction was initiated by the addition of enzyme and the reaction mixture was incubated at 37°C. At appropriate times samples were withdrawn from the reaction mixture and transferred to filter-paper discs (Whatman 3MM; 24 mm diam.). When the filter-paper discs had completely absorbed the reaction-mixture sample (approx. 15 s), the discs were immersed in cold 5% (w/v) trichloroacetic acid for 15 min. The discs were washed twice with fresh portions of cold 5% trichloroacetic acid, once with cold ethanol/ether (1:1, v/v) and finally once with cold ether, each for 15 min at 4°C. After drying in the air, the discs were placed in vials containing the scintillation medium and counted for radioactivity in a liquid-scintillation counter (Beckman model LS200).

To study the effect of an analogue on the extent of aminoacylation of tRNA by an amino acid, a series

of seven standard reaction mixtures was set up, each containing 0.5 nmol of the ^{14}C -labelled amino acid in a final volume of 250 μl . Then 50 μl of a 0.1 M solution of the analogue was added to the first reaction mixture to give an analogue/amino acid molar ratio of 10000:1. Dilutions of the 0.1 M analogue solutions were added to another four of the reaction mixtures to give molar ratios of 1000:1, 100:1, 10:1 and 1:1. The sixth reaction mixture did not contain the analogue and the seventh reaction mixture was a blank containing no tRNA.

At the same time as an analogue was being tested for its effect on the charging of a structurally related amino acid, it was also tested against an unrelated amino acid. This was done to rule out non-specific effects which the inhibitor, particularly at high concentration, might have on the aminoacylation reaction e.g. alteration of the ionic strength of the solution.

Results

Methionine analogues

Analogues that are able to replace methionine in the C-terminal gastrin tetrapeptide are ethionine, norleucine, S-ethylcysteine, norvaline, α -aminobutyric acid, S-methylcysteine and homocysteine. When any of the first three replace the methionine residue the tetrapeptide has full activity, whereas the latter four produce a tetrapeptide that is less active. These analogues were examined for their effect on the charging of tRNA from *E. coli* and rat liver with methionine, and in each case a homologous enzyme preparation was used. Table 1 shows that, in the *E. coli* system, ethionine and homocysteine are the most efficient inhibitors of methionyl-tRNA formation. At an analogue/amino acid molar ratio of 1000:1 L-ethionine and DL-homocysteine inhibit charging

by 84 and 44% respectively and at 10000:1 by 99 and 91% respectively. It should be remembered that the homocysteine is the DL-racemate, so that if only one of the isomers inhibits [D isomers are rarely able to esterify tRNA (Calendar & Berg, 1966)] the ratio for L-homocysteine should in reality be half of the values shown, and, taking this into account, the inhibition becomes 58 and 99% at 1000:1 and 10000:1 ratios respectively.

Norleucine, norvaline and α -aminobutyric acid inhibit methionyl-tRNA formation to a lesser extent and only at the highest analogue/amino acid ratio. S-Methylcysteine and S-ethylcysteine, however, have no effect on the charging reaction.

For the analogues that inhibited the formation of methionyl-tRNA (ethionine, homocysteine, norvaline, norleucine and α -aminobutyric acid) a kinetic assay was performed in which, at analogue concentrations that gave substantial inhibition after 25 min, the reaction was followed at incubation times of 30 s to 60 min. The results (Fig. 1) show that for all the analogues plateaux were reached. Although not shown, this type of experiment was also carried out with the analogues of the other amino acids and in the rat liver system. In all cases the kinetics showed a similar pattern. These results indicate that the analogues are inhibiting the extent of aminoacylation and not just the rate of aminoacylation.

Table 2 shows the effect of ethionine and homocysteine on the aminoacylation of tRNA with other amino acids. Aspartic acid may be considered as a control, and the analogues have no effect on the charging of tRNA with this amino acid, and hence it can be concluded that the analogues, even at the highest concentration, do not inhibit the aminoacylation reaction non-specifically. The effect of these analogues on the charging of tRNA with leucine is interesting. Ethionine inhibits leucyl-tRNA formation at analogue/amino acid molar ratios of 1000:1

Table 1. Effect of analogues on the formation of methionyl-tRNA in an *E. coli* system

Results in this and the following tables (Tables 2–8) are all expressed as pmol of amino acid accepted by one E_{260} unit (measured in 1 ml, 1 cm light-path cell at neutral pH) of mixed crude tRNA. The assays were performed as described in the Materials and Methods section. After 25 min incubation, 200 μl of the 250 μl incubation mixture was transferred to two filter-paper discs pinned together, which were then immersed in cold 5% (w/v) trichloroacetic acid. Some variations seen in the control values are due to assays being performed at different times and with different enzyme preparations.

Ratio (analogue/ amino acid)	Analogue added							
	α -Aminobutyric acid	Ethionine	Norvaline	Norleucine	S-Ethyl- cysteine	S-Methyl- cysteine	DL-Homo- cysteine	L-Homo- cysteine
0	66.0	70.1	70.6	69.8	69.5	69.2	69.6	69.6
1:1	65.7	71.6	71.0	71.0	69.5	70.5	71.9	71.0
10:1	67.2	67.3	68.2	71.6	69.8	69.3	68.9	68.0
100:1	66.3	47.1	70.8	69.8	69.5	70.1	62.3	58.0
1000:1	62.2	11.4	66.6	55.8	68.0	70.2	40.3	29.0
10000:1	39.2	0.7	39.4	31.5	67.3	69.8	6.9	0.0

Fig. 1. Effect of analogues on the kinetics of formation of methionyl-tRNA in an *E. coli* system

The assay was performed as described in the Materials and Methods section. After appropriate incubation times, 50 μ l of a 375 μ l incubation mixture was added to a half filter-paper disc, which was then immersed in cold 5% (w/v) trichloroacetic acid. Analogues present in the reaction mixture at the analogue/methionine molar ratios indicated are shown as follows: α -aminobutyric acid (10000:1), Δ ; ethionine (1000:1), $\blacktriangle$; norvaline (10000:1), $\blacksquare$; norleucine (5000:1), $\circ$; homocysteine (1000:1), $\square$; control (no analogue), Δ .

Table 2. Effect of ethionine and homocysteine on the formation of aspartyl-tRNA and leucyl-tRNA in an *E. coli* system

The assays were performed, and the results are expressed, as described in the legend to Table 1.

Ratio (analogue/ amino acid)	Aspartyl-tRNA		Leucyl-tRNA	
	Ethionine	Homo- cysteine	Ethionine	Homo- cysteine
0	48.9	45.3	38.7	39.8
1:1	48.7	44.0	37.1	39.7
10:1	49.6	46.0	39.2	37.8
100:1	48.3	46.5	31.1	41.0
1000:1	48.0	46.4	10.0	38.9
10000:1	46.9	42.9	0.5	39.4

and 10000:1, the latter giving complete inhibition. DL-Homocysteine, however, has no effect at all on leucyl-tRNA formation. Therefore, whereas for methionine these analogues react in a similar manner, for the charging of tRNA with leucine they behave quite differently. This demonstrates the subtle recognition processes which occur during the aminoacylation reactions of tRNA.

In the rat liver system a very similar picture applies to that found in the *E. coli* system. At an analogue/amino acid molar ratio of 1000:1, L-ethionine and DL-homocysteine both inhibit methionyl-tRNA formation by 50%, and at a ratio of 10000:1 by 90 and 94% respectively. Norleucine, norvaline and α -aminobutyric acid inhibit the methionyl-tRNA formation to a lesser extent at the higher analogue to amino acid ratio. S-Methylcysteine and S-ethylcysteine had no effect on the charging reaction.

As Table 3 shows, the effects of ethionine on the formation of leucyl-tRNA and aspartyl-tRNA in the rat liver system are similar to those observed for the *E. coli* system. However, whereas DL-homocysteine in the rat liver system, like the *E. coli* system, has no effect on the formation of aspartyl-tRNA, rather than inhibiting the function of leucyl-tRNA in the rat liver system DL-homocysteine, in fact, stimulates its formation. This result and similar observations on the formation of Leu-tRNA by other analogues is discussed elsewhere (Old & Jones, 1976).

Phenylalanine analogues

The phenylalanine residue of the gastrin tetrapeptide can be replaced successfully by many analogues.

These include hexahydrophenylalanine, *p*-nitrophenylalanine and *p*-fluorophenylalanine, all of which yield tetrapeptide analogues with activity equivalent to that of the natural tetrapeptide. These analogues were assayed for their ability to inhibit phenylalanyl-tRNA formation in an *E. coli* and a rat liver system. It was also decided to examine the effect of these analogues on tyrosyl-tRNA formation because of the similarity of structure between phenylalanine and tyrosine, although it must be remembered that the aminoacyl-tRNA synthetases clearly distinguish between phenylalanine and tyrosine. Table 4 shows that *L*-*p*-fluorophenylalanine is a good inhibitor of phenylalanyl-tRNA formation, causing 93% inhibition at an [analogue/amino acid molar ratio of 1000:1, and complete inhibition at a molar ratio of 10000:1. *p*-Fluorophenylalanine had no effect on the formation of tyrosyl-tRNA.

L-Hexahydrophenylalanine was found to affect the formation of phenylalanyl-tRNA only at the highest analogue/amino acid molar ratio (10000:1), when 29% inhibition was observed. It had no effect on tyrosyl-tRNA formation. *L*-*p*-Nitrophenylalanine had no effect on either phenylalanyl-tRNA or tyrosyl-tRNA formation.

In the rat liver system as shown in Table 5, *p*-fluorophenylalanine, and to a lesser extent hexahydrophenylalanine, inhibited the formation of phenyl-

alanyl-tRNA. The former inhibited to an extent of 97% at an analogue/amino acid molar ratio of 1000:1, and completely at a molar ratio of 10000:1, whereas hexahydrophenylalanine inhibited only to the extent of 59% at the highest ratio. These results are similar to those obtained in the *E. coli* system, which are shown in Table 4.

None of the analogues inhibits the formation of tyrosyl-tRNA nor does *p*-nitrophenylalanine inhibit

Table 3. Effect of ethionine and homocysteine on the formation of aspartyl-tRNA and leucyl-tRNA in a rat liver system

For details, see Table 1

Ratio (analogue/ amino acid)	Aspartyl-tRNA		Leucyl-tRNA	
	Ethionine	Homo- cysteine*	Ethionine	Homo- cysteine*
0	44.2	30.8	10.8	6.5
1:1	45.8	28.4	11.1	6.8
10:1	45.6	29.3	11.6	7.4
100:1	36.0	26.7	9.5	6.4
1000:1	41.6	28.5	5.5	9.7
10000:1	36.0	27.0	1.1	25.9

* These values are somewhat low because half the usual amount of crude enzyme was used in these assays.

Table 4. Effect of analogues on the formation of phenylalanyl-tRNA and tyrosyl-tRNA in an *E. coli* system

For details, see Table 1.

Ratio (analogue/ amino acid)	Phenylalanyl-tRNA			Tyrosyl-tRNA		
	<i>p</i> -Fluoro- phenylalanine	Hexahydro- phenylalanine	<i>p</i> -Nitro- phenylalanine	<i>p</i> -Fluoro- phenylalanine	Hexahydro- phenylalanine	<i>p</i> -Nitro- phenylalanine
0	32.2	33.8	32.7	17.1	19.6	20.2
1:1	32.2	34.0	32.8	18.8	19.6	18.1
10:1	29.5	34.1	33.0	18.3	19.1	18.7
100:1	16.1	33.6	34.2	19.5	18.8	19.2
1000:1	2.1	31.9	32.6	19.1	19.2	20.1
10000:1	0.2	24.2	33.0	15.3	18.4	19.3

Table 5. Effect of analogues on the formation of phenylalanyl-tRNA and tyrosyl-tRNA in a rat liver system

For details, see Table 1.

Ratio (analogue/ amino acid)	Phenylalanyl-tRNA			Tyrosyl-tRNA		
	<i>p</i> -Fluoro- phenylalanine	Hexahydro- phenylalanine	<i>p</i> -Nitro- phenylalanine	<i>p</i> -Fluoro- phenylalanine	Hexahydro- phenylalanine	<i>p</i> -Nitro- phenylalanine
0	34.0	36.7	31.8	8.5	8.2	8.3
1:1	30.5	35.7	31.4	8.8	10.8	8.4
10:1	17.9	30.9	31.7	4.9	8.9	8.7
100:1	4.9	35.4	30.8	12.0	15.3	15.6
1000:1	0.9	33.7	40.0	15.1	17.1	17.6
10000:1	0.0	14.5	39.5	13.9	16.8	17.5

Table 6. *Effect of analogues on the formation of tryptophyl-tRNA and phenylalanyl-tRNA in an E. coli system*

For details, see Table 1.

Ratio (analogue/amino acid)	Tryptophyl-tRNA		Phenylalanyl-tRNA	
	5-Hydroxytryptophan	5-Methyltryptophan	5-Hydroxytryptophan	5-Methyltryptophan
0	13.6	13.6	32.3	32.3
1:1	14.0	13.9	32.0	32.1
10:1	13.9	14.6	32.0	31.8
100:1	15.4	14.6	31.2	32.0
1000:1	14.7	15.5	30.3	30.8
10000:1	16.3	16.3	32.4	27.6

Table 7. *Effect of analogues on the formation of tryptophyl-tRNA and phenylalanyl-tRNA in a rat liver system*

For details, see Table 1.

Ratio (analogue/amino acid)	Tryptophyl-tRNA		Phenylalanyl-tRNA	
	5-Hydroxytryptophan	5-Methyltryptophan	5-Hydroxytryptophan	5-Methyltryptophan
0	38.5	38.5	39.0	39.0
1:1	36.6	37.1	40.4	37.2
10:1	30.1	38.1	38.1	39.7
100:1	22.4	32.9	41.2	36.0
1000:1	6.4	27.9	40.7	40.4
10000:1	1.5	6.5	43.1	46.0

Table 8. *Effect of L-O-methylthreonine on the formation of isoleucyl-tRNA and threonyl-tRNA in an E. coli and rat liver system*

For details, see Table 1.

Ratio (analogue/ amino acid)	Isoleucyl-tRNA		Threonyl-tRNA	
	<i>E. coli</i>	Rat liver	<i>E. coli</i>	Rat liver
0	24.0	26.7	32.7	48.0
1:1	21.4	24.9	36.2	49.4
10:1	22.1	25.8	37.6	47.5
100:1	17.8	25.6	28.5	33.7
1000:1	2.1	19.7	8.9	10.7
10000:1	0.8	4.5	1.8	1.6

phenylalanyl-tRNA formation. On the contrary, in each of these cases the presence of the analogue at the higher concentrations increases the extent of aminoacyl-tRNA formation. This is an effect similar to that of other analogues on the formation of leucyl-tRNA noted above.

Tryptophan analogues

Among the analogues that are able to replace tryptophan in the gastrin tetrapeptide to yield fully active tetrapeptide analogues are 5-hydroxytrypto-

phan and 5-methyltryptophan. These two analogues were tested in an *E. coli* and a rat liver system for their effect on the formation of tryptophyl-tRNA, and as a control, their effect on phenylalanyl-tRNA formation was also examined. As shown in Table 6 in the *E. coli* system neither of the analogues inhibited the formation of tryptophyl-tRNA or phenylalanyl-tRNA. However, as Table 7 shows a different result was obtained in the rat liver system. Here both 5-hydroxytryptophan and 5-methyltryptophan inhibited the formation of tryptophyl-tRNA, 5-hydroxytryptophan having a somewhat greater effect than the 5-methyl derivative. At an analogue/amino acid molar ratio of 1000:1, 5-hydroxytryptophan gave 83% inhibition and 5-methyltryptophan 25% inhibition, and at a 10000:1 molar ratio they inhibited tryptophyl-tRNA formation by 96 and 82% respectively.

Although for the greater part of this work our choice of analogues was guided by analogue work with the gastrin tetrapeptide, we also examined the affect of L-O-methylthreonine on the formation of isoleucyl-tRNA and threonyl-tRNA. Our interest in this analogue was aroused by results of Smulson & Rabinowitz (1968), who reported that O-methylthreonine inhibited both the rate and extent of isoleucyl-tRNA formation in the *E. coli* system, but only the initial rate in the rat liver. We are aware of no other analogue which is reported to inhibit in this manner. Table 8 shows our results when L-O-

threonine was examined for its effect on the formation of isoleucyl-tRNA and threonyl-tRNA in an *E. coli* and a rat liver system in a manner similar to that described for the testing of the other analogues reported here. Although *O*-methylthreonine inhibits aminoacylation of tRNA in both the *E. coli* and rat liver system at the high analogue/amino acid molar ratios (10^3 and 10^4), at a molar ratio of 100:1 inhibition only occurs in the *E. coli* system. As Smulson & Rabinowitz (1968) used an analogue/amino acid molar ratio of 80:1 in their experiments, our results do not conflict with theirs. However, it appears that *O*-methylthreonine in the rat liver system does inhibit the extent as well as the rate of isoleucyl-tRNA formation, but inhibition of the extent is seen only at high analogue/amino acid ratios.

O-Methylthreonine inhibits the formation of threonyl-tRNA in both the *E. coli* and rat liver systems. Here, in contrast with the differences observed for the effect of this analogue on the isoleucyl-tRNA formation in the two systems, the inhibitory effects on threonyl-tRNA formation in the *E. coli* and rat liver system are found to be similar.

Discussion

The aminoacylation of tRNA involves three steps: (1) the binding of the amino acid and ATP to the enzyme (Allende *et al.*, 1970); (2) the formation of an aminoacyl adenylate complex (Allende & Allende, 1971); (3) the release of pyrophosphate, and the interaction of the aminoacyl adenylate complex with tRNA.

We have sought to determine the structural features of the amino acid side chain which are recognized by its cognate aminoacyl-tRNA synthetase. To do this, the effect on the aminoacylation of tRNA by analogues that differ from the natural amino acid in side-chain structure has been examined.

The choice of analogues used may be made by several criteria, not least by related chemical structure to the natural amino acid. Our choice was guided in this way, but as an additional criterion we chose analogues that have been shown by Morley (1968) and his co-workers to be able to replace successfully amino acids in the 'binding' positions of the C-terminal gastrin tetrapeptide. In choosing these analogues we also sought to discover whether there was a similarity in the recognition of the analogues in the two systems. If this is so, it might indicate that when amino acid side chains are involved in recognition processes, even if they are in totally different environments and are interacting with different proteins, the same structural side-chain features are important.

Examination of the effect of the analogues was assayed by their ability to inhibit the extent of aminoacylation of the tRNA by its cognate amino acid. This was chosen rather than the ATP/PP_i-exchange

assay, which may be used to study the first two steps of the aminoacylation reaction, since in the latter assay, where a crude enzyme preparation is used, it is possible that the analogue may be interacting with more than one aminoacyl-tRNA synthetase, and this would complicate the interpretation of the results. There are three possible ways in which the analogue might be expected to inhibit aminoacylation of tRNA: (a) reversible or (b) irreversible binding to the enzyme without covalent linkage to the tRNA, and (c) reversible binding to the enzyme followed by covalent linkage to the tRNA. (a) should affect the rate, but not the extent, of aminoacylation of the amino acid to its cognate tRNA, whereas (b) and (c) should affect the extent of aminoacylation of tRNA.

The results in Fig. 1 and similar results with the other analogues indicate that it is the extent of aminoacylation that is affected. In studies involving a different assay (J. M. Old & D. S. Jones, unpublished work), it has been shown that the analogues described in the present paper inhibit aminoacylation of tRNA by mechanism (c).

Of the seven analogues listed for their effect on formation of methionyl-tRNA, two, ethionine and homocysteine, are found to be relatively good inhibitors; norleucine, norvaline and α -aminobutyric acid inhibit only poorly; *S*-methylcysteine and *S*-ethylcysteine have no effect at all. Ethionine and norleucine are known to inhibit the growth of *E. coli* and are incorporated into protein in place of methionine (Richmond, 1962). Further, these analogues have been known to substitute for methionine in the aminoacylation of tRNA in an *E. coli* system (Trupin *et al.*, 1966). Our results support these findings and would suggest that homocysteine can equally replace methionine. The results shown here also suggest that what has been found in *E. coli* holds equally true in rat liver.

Examination of the structures of the five analogues that inhibit methionyl-tRNA formation shows variation in the length of the side chain, so that for binding to take place at the enzyme site, size is not absolutely critical. The best two inhibitors, ethionine and homocysteine, have a sulphur atom in a position similar to that in methionine, whereas in the analogues that do not inhibit, namely *S*-methylcysteine and *S*-ethylcysteine, the sulphur atom is in a position nearer by one atom to the amino acid backbone. This suggests that there may well be a specific interaction between the sulphur atom and a group in a rigid position at the active site of the enzyme. A change in the position of the sulphur atom in the analogue prevents the analogue from adopting a position that presents the sulphur atom to its specific site and allows reaction of the amino acid carboxyl group.

Since the results obtained with these amino acid analogues in the rat liver system are essentially the same as those obtained with the *E. coli* system, it

Table 9. Comparison of the effects of amino acid analogues on gastrin activity and the aminoacylation of tRNA

+++ represents good inhibition; + represents inhibition only at an analogue/amino acid ratio molar of 10000:1; - represents no inhibition.

	Inhibition of aminoacyl-tRNA formation					
Gastrin tetrapeptide	Methionine		Phenylalanine		Tryptophan	
As active tetrapeptide	Ethionine	+++	<i>p</i> -Fluorophenylalanine	+++	5-Hydroxytryptophan	
	Norleucine	+	<i>p</i> -Nitrophenylalanine	—	Rat liver	+++
	<i>S</i> -Ethylcysteine	—	Hexahydrophenylalanine	+	<i>E. coli</i>	—
Less active tetrapeptide	Norvaline	+			5-Methyltryptophan	
	α -Aminobutyric acid	+			Rat liver	+++
	<i>S</i> -Methylcysteine	—			<i>E. coli</i>	—
	Homocysteine	+++				

would appear that the structural requirements of the amino acid side chain for recognition by the rat liver and *E. coli* methionyl-tRNA synthetases are the same, and therefore it may be that the parts of these synthetases involved in this recognition have similar features.

The three analogues tested for their effect on the formation of phenylalanyl-tRNA show three different effects that are similar in both the rat liver and *E. coli* systems. *p*-Fluorophenylalanine is found to be a good inhibitor of phenylalanyl-tRNA formation, which supports results obtained by Santi & Danenberg (1971), who showed that *p*-fluorophenylalanine supported ATP/PP_i exchange in the presence of pure *E. coli* phenylalanyl-tRNA synthetase. Hexahydrophenylalanine only inhibited the formation of the phenylalanyl-tRNA poorly and *p*-nitrophenylalanine had no effect on the aminoacylation reaction. The difference in result between fluorophenylalanine and nitrophenylalanine suggests that the size of the side chain is important. A fluorine substituent is of similar size to that of hydrogen and is a permissible replacement. However, the replacement of hydrogen by bulkier substituents such as a hydroxyl group (the natural amino acid tyrosine) or a nitro group is not permissible. Also it should be considered that both of these groups introduce a polarity into the side chain although with opposite mesomeric effects. The finding that hexahydrophenylalanine inhibits to only a slight extent suggests that the planar shape and electron density of the benzene ring are important in the recognition of phenylalanine. Hexahydrophenylalanine possesses a cyclohexane ring that is not planar, nor does it possess an electron cloud.

These few results suggest therefore that for both the *E. coli* and the rat liver phenylalanyl-tRNA synthetases the parts of the enzyme involved in binding the amino acid are only able to recognize a non-polar planar benzene ring in the side-chain position of the amino acid. This conclusion is consistent with that of Santi & Danenberg (1971), who, by using a wide range of analogues of phenylalanine in the ATP/PP_i-exchange reaction, concluded that the benzene ring

of phenylalanine is the primary site of recognition and also one of the two major binding loci (the other being the amino group).

The results obtained with analogues examined for their effect on the formation of tryptophyl-tRNA are distinguished from those described previously by the fact that differences are seen between the *E. coli* and rat liver systems. In the *E. coli* system it is found that neither 5-methyl- nor 5-hydroxy-tryptophan has any effect on the formation of tryptophyl-tRNA, and it therefore appears that the *E. coli* tryptophyl-tRNA synthetase is unable to accommodate additional groups in the indole-ring system. By contrast, however, the rat liver synthetase is able to accommodate the 5-hydroxyl group and to a lesser extent the 5-methyl group. These results would imply either that if the two synthetases are similar in structure at the amino acid recognition site the rat liver synthetase allows a looser fit, or that the recognition sites of the *E. coli* and rat liver tryptophyl-tRNA synthetases are not comparable.

As stated above, the choice of analogues in the present study was guided in part by successful replacement of amino acid residues by analogues in the gastrin tetrapeptide. A comparison of the results described in the present paper with those obtained for gastrin is summarized in Table 9, which shows that it is difficult to draw comparisons. Indeed, some analogues that replace amino acids very well in the gastrin tetrapeptides are found to have no effect on the aminoacylation reaction, e.g. *S*-ethylcysteine, *p*-nitrophenylalanine and 5-hydroxytryptophan (*E. coli*), whereas analogues such as homocysteine and 5-methyltryptophan (rat liver) show good inhibition of aminoacyl-tRNA formation but were less effective when substituted into the gastrin tetrapeptide. It appears, therefore, that the amino acid side chains are recognized in a different manner by the gastrin-receptor binding site and the synthetase binding sites.

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