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Differential Coupling Efficiency of Chemically Activated Amino Acid to tRNA

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Summary. Interaction based on possible chemical affinity of an amino acid for tRNA was examined as a model for the aminoacylation of primitive tRNA without aid of an enzyme system. Two types of reaction were carried out and compared. One was the acyl linkage of amino acid to the 5′-terminal phosphate of a tRNA activated as an imidazolide. The other was the incorporation of an amino acid activated as an imidazolide into 2′(3′)-hydroxyl groups of intact tRNA. Both types of reaction indicated that none of the amino acids tested had any selectivity for the tRNAs examined. However, the rates of reaction with a given tRNA were different among amino acids. In the second type of reaction, amino acids were found mainly at loop-out regions of tRNA, but not at either its 5′- or 3′-terminal sites

Key words: Primitive tRNA — tRNA — Aminoacyl imidazole — Aminoacyl tRNA — Amino acid charging — Prebiotic — Amino acid–polynucleotide interaction

Introduction

In modern organisms, specific interaction between an amino acid species and its cognate tRNA is catalyzed by the corresponding aminoacyl-tRNA synthetase. At the primitive stage of the origin of life, however, we would not expect the existence of such a specific catalytic system. It has been assumed that nucleotide or polynucleotide directly discriminated a specific amino acid at such an early stage of biochemical evolution (Ishigami et al. 1977). Crothers et al. (1972) pointed out the existence of a relationship between the fourth nucleotide from the 3′ end of tRNA and the physicochemical nature of its cognate amino acid. They also postulated that this relation reflected a primitive categorization of various amino acids retained in the modern organism. On the other hand, Weber and Lacey (1978) reported a correlation between the hydrophobicity of a dinucleotide in the anticodon sequence and that of the cognate amino acid. Weber and Fox (1973) reported that transfer of N-acetylaminoacyl-imidazoles to the 2′-hydroxyl groups of the four kinds of homopolyribonucleotides [poly(A), poly(G), poly(C), and poly(U)] occurred in the highest yield with poly(U). N-acetylglycyladenylate anhydride also transferred to poly(U) in the highest yield in the presence of imidazole (White et al. 1973). Weber and Lacey (1975) reported that unprotected phenylalanyladenylate could be transferred to homopolyribonucleotides by using imidazole as a catalyst. The transfer reaction was again most effective for poly(U), and the reaction to a poly(A)–poly(U) double helix was negligible.

In the present paper, specific interaction between polynucleotide and amino acid was tested using bacterial tRNA as a model polyribonucleotide. In one series of experiments, amino acids were chemically attached to the 5′-terminal phosphate of tRNA by the method of Lohrman et al. (1975). In another series, they were attached to the 2′(3′)-hydroxyl group(s) of tRNA by the method of Gottikh et al. (1970). In the first type of experiment (Fig. 1A), only

Abbreviations: AA-Im, aminoacyl imidazolide; 2′(3′)-O-AA-tRNA, 2′(3′)-O-aminoacyl-tRNA; 5′-O-AA-tRNA, phosphate ester of amino acid at the 5′-terminal phosphate of tRNA; CDI, N,N′-carbonyldiimidazole; DMF, dimethylformamide; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; Im-tRNA, 5′-imidazolylphosphonate of tRNA

One A_{260} unit is defined as an amount of material which gives an absorption of 1.0 at 260 nm when dissolved in 1 ml water and measured with a 1-cm light path

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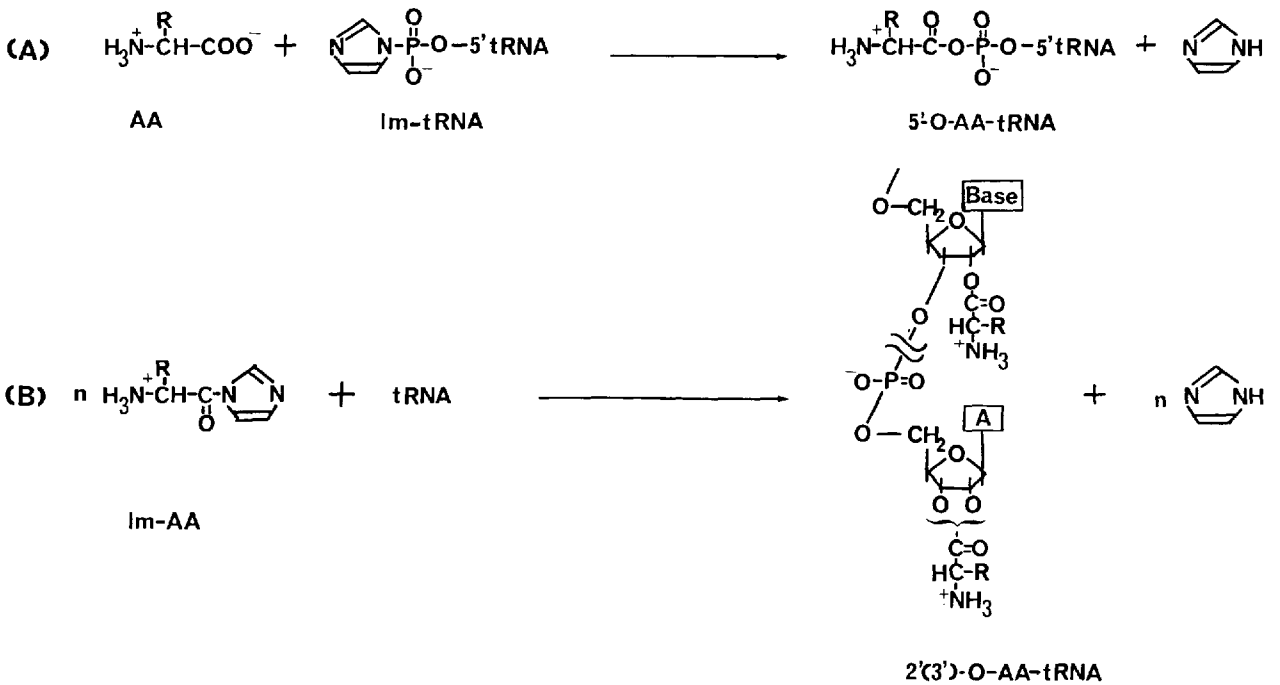

Fig. 1. Two types of reaction between amino acid and tRNA. **A** Amino acid reacts with activated tRNA (Im-tRNA) to form 5'-O-AA-tRNA. **B** Activated amino acid (Im-AA) reacts with the 2'(3')-hydroxyl group(s) of tRNA to form 2'(3')-O-AA-tRNA

5'-terminal phosphate of tRNA could accept the amino acid. In the second type of experiment (Fig. 1B), the activated amino acids are probably bound to the 2'(3')-hydroxyl group(s) of each nucleotide in the tRNA sequence.

Materials and Methods

^{3}H-labeled amino acids, Phe (18 Ci/mmol), Gly (23 Ci/mmol), and Val (33 Ci/mmol); and ^{14}C-labeled amino acids, Ser (171 mCi/mmol), Thr (238 mCi/mmol), and Val (270 mCi/mmol), were purchased from New England Nuclear and Radiochemical Center (Amersham, England), respectively. Unlabeled amino acids were from Kyowa Hakko Kogyo Co., Ltd.; N,N'-carbonyldiimidazole (CDI) was from Sigma Chemical; and triphenylphosphine, cetyltrimethylammonium bromide, and triethylamine were from Wako Pure Chemical Industry, Ltd. 2,2'-Dipyridyldisulfide was from Tokyo Chemical Industry Co., Ltd.; and trifluoroacetic acid (TFA) and imidazole were from Nakarai Chemical, Ltd. Dimethylsulfoxide (DMSO) and dimethylformamide (DMF) were dried with synthetic Zeolite (CA-3, short 14–30 mesh), which was purchased from Wako Pure Chemical Industry, Ltd. Brewer's yeast $tRNA^{Phe}$ was purchased from Boehringer Mannheim GmbH. tRNA from *Bacillus subtilis* W 168 was prepared as described previously (Hasegawa and Ishikura 1978). Crude tRNA was first fractionated by Sepharose-4B column chromatography (Holmes et al. 1975). Purified $tRNA^{Thr}$ was prepared by the successive use of BD-cellulose (Gillam et al. 1967) and RPC-5 chromatography (Vold 1973). *Escherichia coli* $tRNA^{Ser}$ was kindly given by Dr. Y. Yamada, Jichi Medical School.

Determination of Aminoacyl Imidazolide

The amount of aminoacyl imidazolide (AA-Im) was determined by the absorption at 245 nm by the method of Jencks and Carriuolo (1959) as follows: 0.5 mmol TFA salt of amino acid was dissolved in 250 μl DMF. A portion of this solution (10 μl) was added to CDI solution (20 μmol/10 μl DMF). After the sample was stirred vigorously for 3 min, 5 μl of the reaction mixture was poured into 3 ml 0.5 M imidazole–HCl buffer (pH 7.0) and the time course of the absorbance shift at 245 nm was examined.

Formation of 5'-O-AA-tRNA

The 5'-phosphate ester of amino acid at the 5'-terminal phosphate of tRNA (5'-O-AA-tRNA) was prepared from amino acid and the 5'-imidazolylphosphonate of tRNA (Im-tRNA). Im-tRNA was prepared by the modified method of Mukaiyama and Hashimoto (1971) which was originally used for synthesis of adenosine-5'-imidazolylphosphonate. Crude tRNA (40 A_{260} units) was modified to the cetyltrimethylammonium salt to increase its solubility in organic solvent (Johes 1953). The crude tRNA was dissolved in 100 μl DMSO containing 132 μmol imidazole and 56 μmol dipyridyldisulfide, and 56 μmol of triphenylphosphine was added to the DMSO solution. To stabilize the reaction products 30 μl triethylamine was added. The mixture was shaken at room temperature overnight. The reaction mixture was poured into a mixture of acetone (5 ml), ether (2.5 ml), and triethylamine (0.25 ml) containing $NaClO_4$ (9 mg) under vigorous stirring. The resulting precipitate of Im-tRNA was collected by centrifugation, washed with acetone and ether, and then dried over P_2O_5 and solid NaOH.

5'-O-AA-tRNA was prepared from Im-tRNA and amino acid by the modified method of Lohrman et al. (1975). [^{14}C]Ser (100 nmol) plus [^{3}H]Phe (10 nmol) in 700-μl solution was added to 40 A_{260} units of Im-tRNA and the mixture adjusted to pH 4.0 with 0.1 N HCl. This optimum pH was determined by preliminary experimentation. This reaction mixture was lyophilized and kept at 4°C for 24 h over P_2O_5 in vacuo. The specimen was dissolved in 200 μl 10 mM sodium acetate buffer (pH 4.5) and applied to a Sepharose-4B column, as described by Holmes et al. (1975). For the assay by filter paper disk, 1 nmol [^{3}H]Phe, 10 nmol [^{14}C]Ser, and 4 A_{260} units tRNA were used as starting materials.

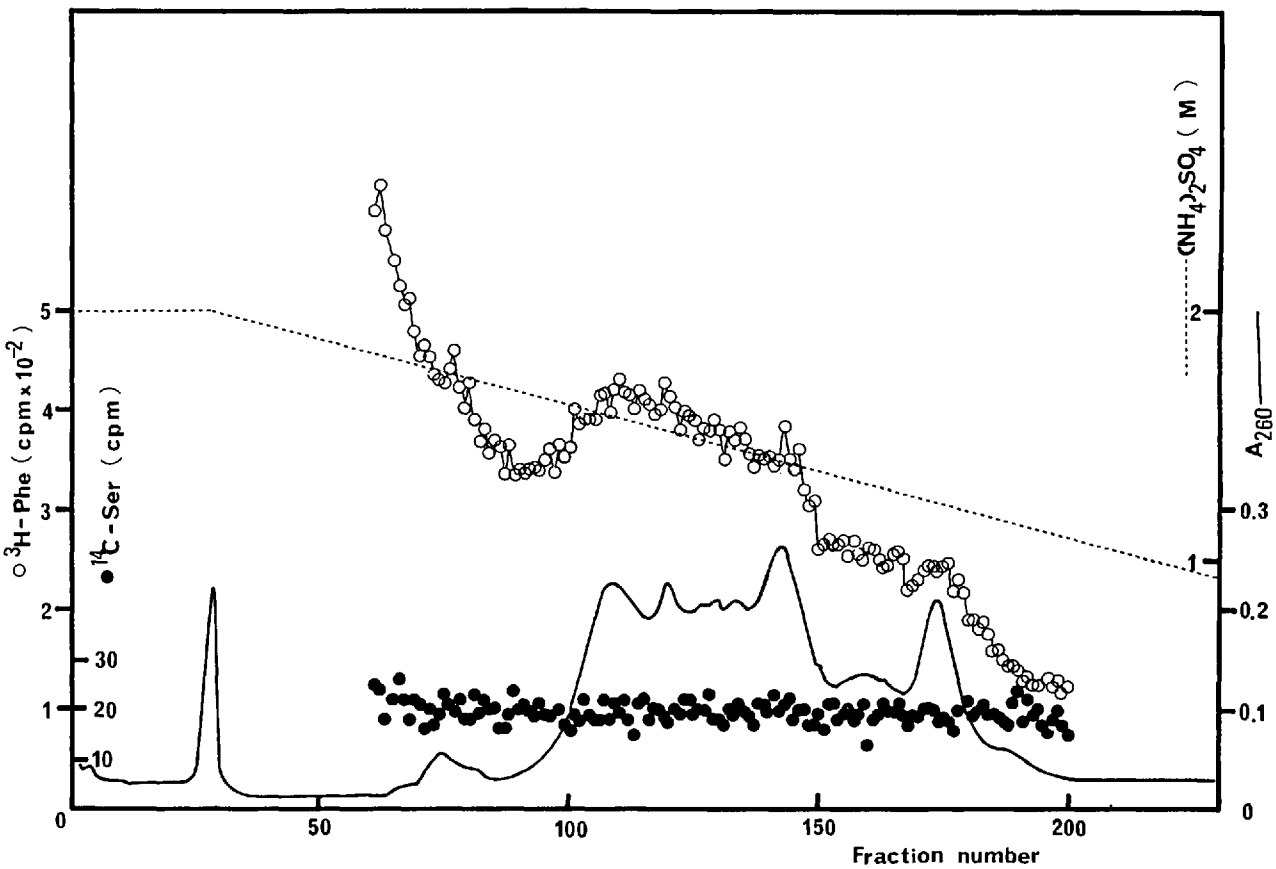

Fig. 2. Analysis of 5'-O-AA-tRNA by Sepharose-4B column chromatography (1 × 33 cm). [^{14}C]Ser (100 nmol) or [^{3}H]Phe (10 nmol) and 40 A_{260} units of Im-tRNAs were reacted as described in Materials and Methods. After the reaction, the reactant was dissolved in 10 mM sodium acetate buffer (pH 4.5) containing 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA), and 2 M ammonium sulfate. Elution was carried out at 4°C with a reverse gradient of ammonium sulfate from 2 to 0 M. The flow rate was 5 ml/h. Each fraction contained 1 ml eluent and was assayed for its absorbance at 260 nm. A portion (0.5 ml) of each fraction was diluted to 2.3 ml with H_2O and its radioactivity in 10 ml Aquazol-II counted on a liquid scintillation counter

Table 1. Yields of 5'-O-AA-tRNAs from amino acid and Im-tRNA

tRNA	Amino acid	
	Phe	Ser
Phe	0.33	1.4
Ser	0.38	1.9
Thr	0.34	1.9
Gly	0.28	1.9

5'-O-AA-tRNA was obtained from [^{3}H]Phe (1 nmol) or [^{14}C]Ser (10 nmol) and 40 A_{260} units of tRNA, respectively. Yield was calculated by taking the radioactivity of the products as a percentage of the total amino acid put into the reaction mixture. Only $tRNA^{Thr}$ from *Bacillus subtilis* was a pure preparation. The other tRNAs were partially purified specimens

Formation of 2'(3')-O-AA-tRNA

2'(3')-O-aminoacyl tRNA [2'(3')-O-AA-tRNA] was prepared by the method of Gottikh et al. (1970), which had originally been applied to the synthesis of 2'(3')-O-aminoacyl mononucleotide. Amino acid was activated as the aminoacyl imidazolide and transferred to the 2'(3')-hydroxyl groups of the ribose moiety in tRNA as follows: 10 nmol [^{14}C]Ser and 10 nmol [^{3}H]Phe were dried, dissolved in 100 μl TFA, and dried again. The trifluoroacetate salt of the amino acid was kept overnight in a desiccator in the presence of P_2O_5 and solid NaOH to remove excess TFA. The trifluoroacetate salt of the amino acid was dissolved in 2 μl DMF containing 200 nmol CDI and agitated for 3 min. Then 40 A_{260} units of crude tRNA in 20 μl 0.5 M imidazole–HCl buffer (pH 7.0) was added to the reaction mixture. This was kept for 3 h at room temperature. The resulting 2'(3')-O-AA-tRNA was precipitated by addition of two volumes of ethanol and collected by centrifugation. The precipitate was dissolved in 200 μl 10 mM sodium acetate buffer (pH 4.5) and the yield of 2'(3')-O-AA-tRNA analyzed by Sepharose-4B column chromatography. For the assay by filter paper disk, 0.5 nmol of each amino acid and 2 A_{260} units of each tRNA were used as starting materials.

Determination of Aminoacyl-tRNA by Filter Paper Disk Assay

A 20-μl portion of the reaction mixture containing 2'(3')-O-AA-tRNA was diluted with imidazole–HCl buffer (pH 7.0). The dried reaction mixture containing 5'-O-AA-tRNA was diluted with 200 μl 10 mM sodium acetate buffer (pH 4.5) containing 2 M ammonium sulfate. A portion of this mixture (50 μl) was put on a filter paper disk (Whatmann 3 MM, 2.4 cm). Unreacted amino acid was washed off by gently shaking twice with cold 5% trichloroacetic acid for 15 min. After the paper disks were dried, their radioactivity was measured by a toluene-based (0.4% PPO, 0.01% POPOP) liquid scintillation counter.

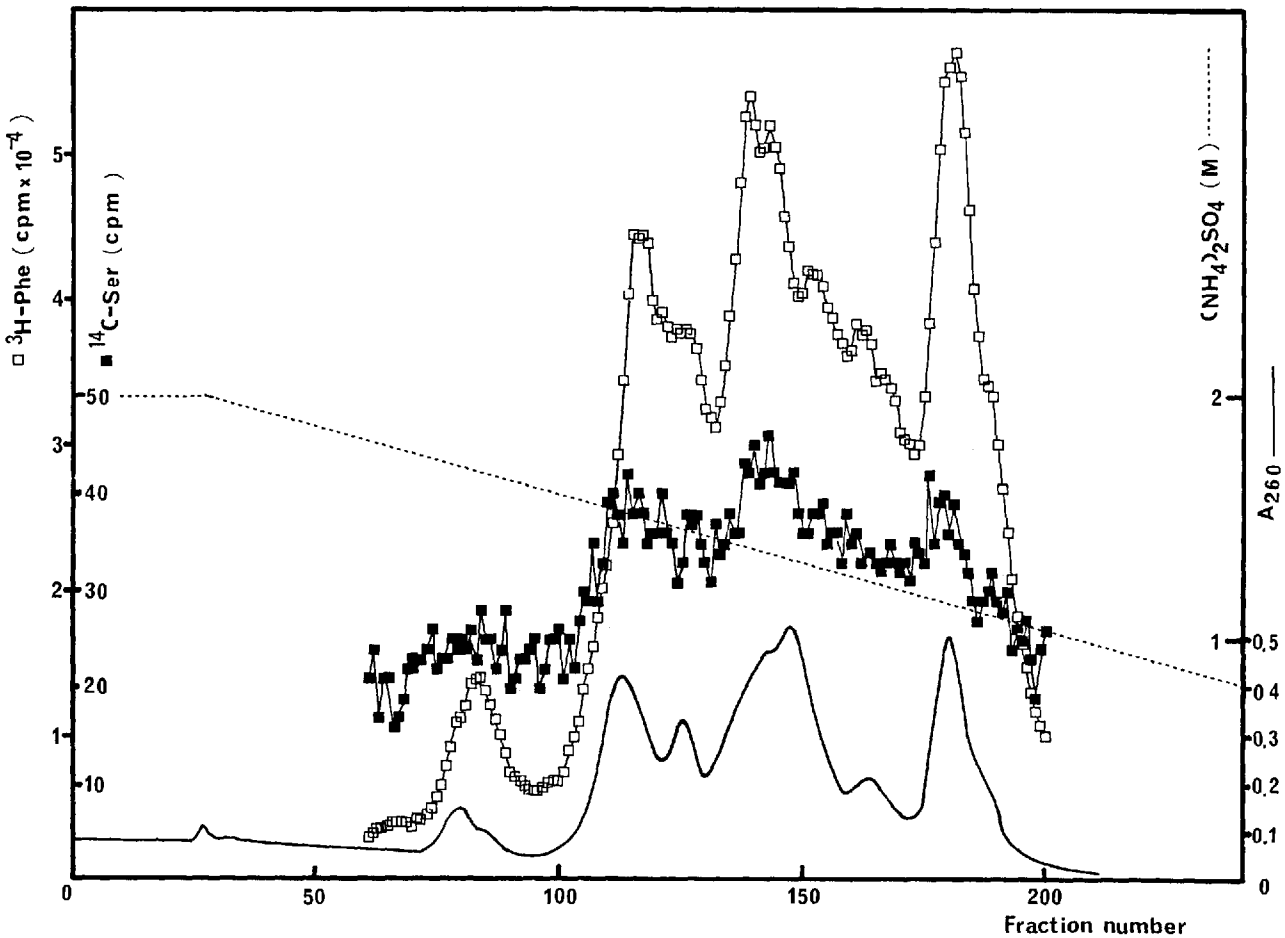

Fig. 3. Analysis of Sepharose-4B column chromatography of 2′(3′)-O-AA-tRNAs. The reaction mixture contained [^{14}C]Ser (10 nmol) or [^{3}H]Phe (10 nmol) and 40 A_{260} units of crude tRNA. The conditions of chromatography were as described in Fig. 2. The reaction conditions are described in Materials and Methods

Determination of Amino Acid Binding Region in tRNA

The aminoacylated region in the total 2′(3′)-hydroxyl groups of tRNA was determined as follows. Purified $tRNA^{Thr}$ from *Bacillus subtilis* was aminoacylated as described above. The modified $tRNA^{Thr}$ was digested with RNase T_1 and analyzed by two-dimensional electrophoresis (Sanger et al. 1965). Each spot of oligonucleotide was cut out and immersed in toluene scintillator solution and the radioactivity counted on a liquid scintillation counter.

Results and Discussion

Aminoacylation on 5′-Phosphate of tRNA

Aminoacylated product was analyzed by Sepharose-4B column chromatography (Fig. 2). No significant incorporation of [^{14}C]Ser was detected, whereas radioactivity of [^{3}H]Phe was observed along with various tRNA fractions. Analysis by filter paper disk, however, indicated no differences in degree of aminoacylation among the various kinds of tRNA, but the relative yield of Phe bound to tRNA was more than that of Ser, because ten times as much Ser was added as Phe (Table 1). Tailing of the radioactivity in the chromatographic pattern suggests that 5′-O-AA-tRNA may be labile and was hydrolyzed to amino acid and tRNA during elution.

Aminoacylation on 2′(3′)-Hydroxyl Groups of tRNA

Aminoacylation products on 2′(3′)-hydroxyl groups of tRNA were also analyzed by column chromatography on Sepharose-4B (Fig. 3). It was found that the distribution of radioactivity of [^{14}C]Ser roughly paralleled the elution curve of tRNA, suggesting nonselective incorporation of the amino acids. The distribution of peaks of incorporated [^{3}H]Phe subtly but definitely corresponded to the tRNA elution curve. In general, the peaks of [^{3}H]Phe were slightly retarded compared with the peaks of absorbance. This disagreement may come from the difference in mobility between free tRNA and tRNA charged with a hydrophobic amino acid such as Phe. This phenomenon was also observed with purified $tRNA^{Thr}$

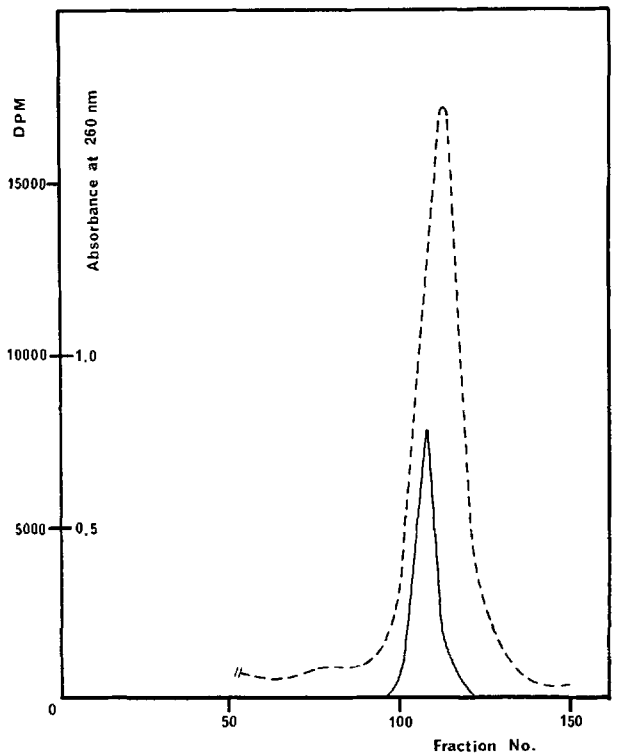

Fig. 4. Analysis of Sepharose-4B column chromatography of 2′(3′)-O-[^{3}H]Phe-tRNA^{Thr} and intact tRNA^{Thr} from *Bacillus subtilis*. Conditions of chromatography were as described in Fig. 2. ——, Ultraviolet absorbance at 260 nm of intact tRNA^{Thr} from *Bacillus subtilis*; – – –, radioactivity (DPM) of 2′(3′)-O-[^{3}H]Phe-tRNA^{Thr}.

Table 2. Yields of 2′(3′)-O-AA-tRNAs

	Amino acid									
	Phe		Ser		Thr		Gly		Val	
tRNA	−Mg	+Mg	−Mg	+Mg	−Mg	+Mg	−Mg	+Mg	−Mg	+Mg
Phe[1]	1.0	2.3	0.1	0.2	0.2	0.4	3.8	3.9	1.4	1.4
Ser[2]	2.9	3.9	0.1	0.5	0.2	0.6	7.8	6.6	3.0	1.6
Thr	2.7	4.3	0.2	0.9	0.2	0.6	7.9	7.0	2.6	2.3
Phe	3.0	4.2	0.2	0.5	0.4	0.5	9.3	6.8	3.3	1.7
Ser	2.8	4.2	0.2	0.6	0.1	0.6	7.6	6.4	3.3	1.8
Gly	2.6	4.0	0.2	0.7	0.5	0.6	8.8	6.7	3.2	1.9
Val	3.5	4.6	0.4	1.0	0.3	0.6	8.3	6.8	3.3	2.0
Arg	3.0	4.1	0.4	1.0	0.4	0.6	9.3	6.8	3.5	1.8
Pro	2.9	4.1	0.3	0.9	0.2	0.6	7.3	6.4	3.3	1.7

2′(3′)-O-AA-tRNA was prepared from each amino acid (0.5 nmol) and tRNA (2 A_{260} units). Yield was calculated as described in Table 1. tRNA^{Thr} (*Bacillus subtilis*), [1]tRNA^{Phe} (Brewer's yeast), and [2]tRNA^{Ser} (*Escherichia coli*) were pure preparations. The other preparations were partially purified specimens from *Bacillus subtilis*

and 2′(3′)-O-Phe-tRNA^{Thr}. 2′(3′)-O-Phe-tRNA^{Thr} eluted in slightly later fractions than intact tRNA^{Thr} (Fig. 4).

No significant difference was observed between the yields of the various 2′(3′)-O-AA-tRNAs for a given amino acid when they were analyzed by the

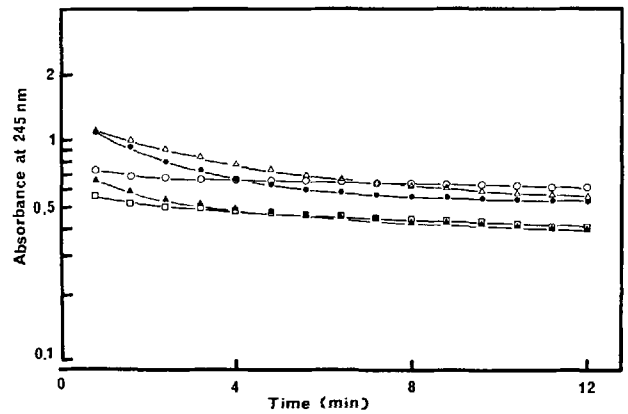

Fig. 5. The rate of hydrolysis of AA-Im. Each amino acid was imidazolized with CDI as described in Materials and Methods. After 3 min of reaction, 5 μl reactant was transferred to 3 ml 0.5 M imidazole buffer (pH 7.0). Spontaneous decay of excess CDI occurred in the initial 1 min. Phe (○), Gly (●), Val (△), Ser (□), Thr (▲)

filter paper disk method (Table 2). However, the yields of 2′(3′)-O-AA-tRNA were different when different amino acids were used as the starting ligands. The yield of 2′(3′)-O-AA-tRNA decreased in the order Gly > Val > Phe > Ser ≃ Thr. A similar difference was observed in 5′-O-AA-tRNA formation experiments. Ehler and Orgel (1976) indicated that N,N′-carbonyldiimidazole reacts with amino acid in aqueous imidazole buffer to yield N-[imidazolyl-(1)-carbonyl]-amino acids, which cyclize to N-carboxyanhydride. Aminoacyl imidazolide derived from N-carboxyanhydride probably transfers to tRNA. The cyclization of N-[imidazolyl-(1)-carbonyl]-L-serine to L-2-oxo-oxazolidine-5-carboxylic acid (Ehler et al. 1977) may compete with tRNA acylation and result in the low yield for this amino acid. A similar result was obtained for Thr. The lower yield of Phe-tRNA compared with that of Gly-tRNA may be due to the more rapid competing hydrolysis of Phe-Im than of Gly-Im, as reported by Weber and Fox (1973). Preincubation with $MgCl_2$ (10 mM) had some effect on the yields of the 2′(3′)-O-AA-tRNAs of Phe, Ser, and Thr, but no effect on 2′(3′)-O-AA-tRNA of Gly and Val. This can be explained on the basis that $MgCl_2$ has some effect on the structure of tRNA and changes the correlation for some kinds of amino acids. The yield of AA-Im was not different among the various amino acids used (Fig. 5). Thus, the different yields of amino acids bound to tRNA seem not to depend on the different yields of AA-Ims, but on the binding rates of the activated amino acids (AA-Im) to tRNA.

To elucidate the reacting site(s) of the tRNA with AA-Im, purified tRNA^{Thr} from *Bacillus subtilis*, the nucleotide sequence of which had already been determined (Hasegawa and Ishikura 1978) was aminoacylated. The resulting 2′(3′)-O-[^{3}H]Phe-tRNA^{Thr}

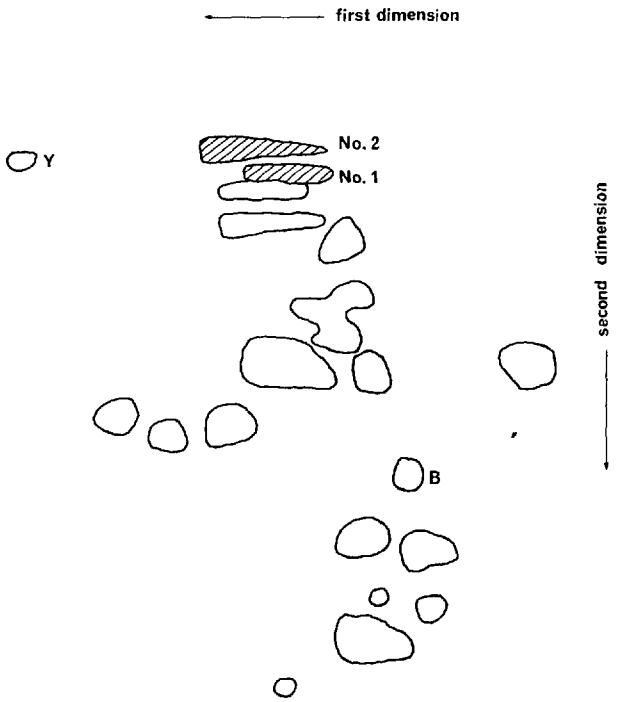

Fig. 6. Two-dimensional fingerprint analysis of complete digestion products of 2′(3′)-O-[^{3}H]Phe-tRNA^{Thr} with RNase T_1. First dimension: cellulose acetate, 5% acetic acid (pH 3.5) containing 7 M urea, 5 kV per 76 cm, 45 min; second dimension: DEAE-cellulose paper, 7% formic acid (pH 1.9), 980 V per 60 cm, 5 h. Radioactivity was observed in the hatched spots (nos. 1 and 2). B and Y are xylene cyanol FF and Orange G markers, respectively

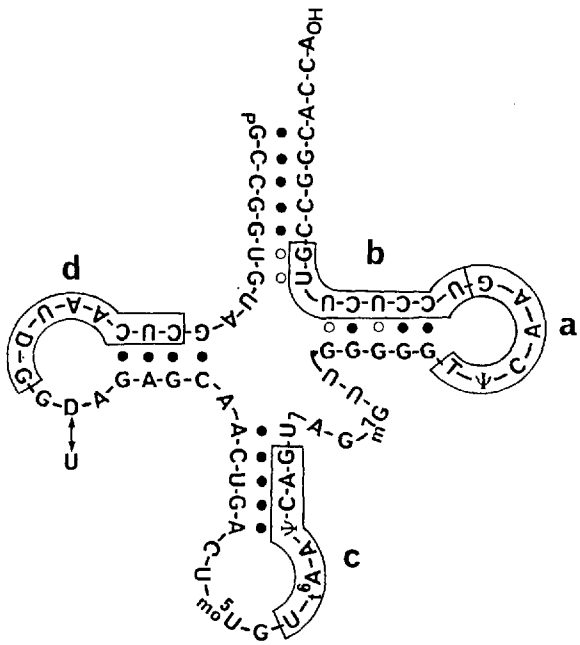

Fig. 7. Aminoacylated oligonucleotides in tRNA^{Thr} from *Bacillus subtilis* depicted in cloverleaf model. **a** corresponds to spot no. 1 of Fig. 6, and **b**, **c**, and/or **d** correspond to spot no. 2

was digested with RNase T_1 and the fragments separated by two-dimensional electrophoresis. Each spot was cut out and counted in a toluene-based liquid scintillation counter (Fig. 6). The nucleotide compositions of spots no. 1 and 2 were confirmed by two-dimensional thin-layer chromatography (Hasegawa and Ishikura 1978). [^{3}H]Phe bound to oligonucleotide no. 1 of Fig. 6 corresponding to molecular region (a) in Fig. 7. [^{3}H]Phe also bound to oligonucleotide no. 2 of Fig. 6, corresponding to molecular regions (b), (c), or (d) in Fig. 7. This result suggests that [^{3}H]Phe has no specific affinity for the amino acid acceptor stem, the anticodon, or the disciminator base of the tRNA. It mainly bound to the loop-out regions. This result seems quite reasonable, since the transfer of N-(aminoacyl)-imidazole to a poly(A)–poly(U) double helix was negligible (Weber and Lacey 1975).

In conclusion, 5′-O-AA-tRNA and 2′(3′)-O-AA-tRNA were chemically formed in the reaction between amino acid and tRNA when either of the two reactants was chemically activated. The yields of various aminoacyl-tRNAs thus obtained were not different for different kinds of tRNAs, but were different for different kinds of amino acids. [^{3}H]-Phe bound to 2′(3′)-hydroxyl groups of the tRNA was located only in the loop-out regions.

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