coli exogenous suppressor tRNA has been shown to be functional (Yamamoto *et al.*, 1971).

The mechanism(s) of inhibition of ovalbumin synthesis and the accumulation of other protein(s) under the influence of hepatoma tRNA is obscure: studies with an isolated protein synthesizing system might provide an insight. It would also be of interest to use purified novel isoacceptor tRNAs from Novikoff hepatoma (Baliga *et al.*, 1969) in ovalbumin synthesis to ascertain whether they simulate the effects of the total population of tRNAs from that source.

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Recognition of the 3′ Terminus of 2′-*O*-Aminoacyl Transfer Ribonucleic Acid by the Acceptor Site of Ribosomal Peptidyltransferase†

David Ringer, Kevin Quiggle, and Stanislav Chládek*

ABSTRACT: The interaction of the 3′ terminus of 2′- and 3′-*O*-aminoacyl-tRNA with the peptidyltransferase A site of *Escherichia coli* ribosomes has been studied using the following aminoacyl oligonucleotides as models of the 3′ terminus of AA-tRNA: C-A-Phe, C-A(2′Phe)H, C-A(2′H)Phe, C-A(2′Phe)Me, C-A(2′Me)Phe, C-A(2′Gly)H, and C-A(2′H)Gly. The transfer of Ac-[^{14}C]Phe from the Ac-[^{14}C]Phe-tRNA · poly(U) · 70S ribosome complex to puromycin (10^{-4} and 10^{-5} M) was inhibited by C-A-Phe, C-A(2′Phe)H, C-A(2′H)Phe, C-A(2′Gly)H, and C-A(2′H)Gly. Kinetic analysis of the inhibition of Ac-[^{14}C]Phe-puromycin formation by C-A(2′Phe)H failed to show simple competitive inhibition. Binding of C-A-C-C-A-[^{14}C]Phe to 70S ribosomes in the presence of an excess of deacylated tRNA was also inhibited by C-A-Phe, C-A(2′Phe)H, C-A(2′H)Phe, C-A(2′Phe)Me, and C-A(2′Me)Phe. It appears that the acceptor site of peptidyltransferase can recognize the 3′ terminus of either 2′- or 3′-*O*-AA-tRNA, with preference for the 2′ isomer. It therefore follows that 2′-*O*-AA-tRNA may be bound to ribosomes prior to peptide bond formation and that 3′-*O*-AA-tRNA, which is used exclusively by peptidyltransferase as an acceptor, is supplied by 2′ → 3′ transacylation occurring at the peptidyltransferase A site.

It was predicted by Zamecnik (1962) that 2′-*O*-aminoacyl-tRNA may be formed by enzymic aminoacylation of tRNA and that 3′-*O*-aminoacyl-tRNA might be used in later stages of protein biosynthesis. Studies of Wolfenden *et al.* (1964), McLaughlin and Ingram (1965), and Griffin *et al.* (1966) have demonstrated the extremely rapid 2′ ⇄ 3′ isomerization of the aminoacyl group on the terminal adenosine unit of tRNA. The finding of Nathans and Neidle (1963) that puromycin, a nonisomerizable analog of 3′-*O*-aminoacyl-tRNA, inhibits polypeptide synthesis, whereas its 2′ isomer is inactive, has often been referred to by various authors as proof that only 3′-*O*-aminoacyl-tRNA can participate in protein synthesis at the ribosomal level. Indeed, Phe-tRNA-C-C-3′-dA (the 2′ ester)[1] prepared by

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Sprinzl and Cramer (1973) did not appear to participate in poly(U)-dependent polyphenylalanine synthesis, and this was interpreted by the authors to be a result of the inability of Phe-tRNA-C-C-3′-dA to undergo the necessary 2′ → 3′ transacylation.

By utilizing relatively simple nonisomerizable nucleoside and oligonucleotide models of the acceptor terminus of 3′-*O*-aminoacyl-tRNA and its 2′ isomer, we were able to demonstrate that 3′-*O*-aminoacyl esters act as acceptors at the peptidyltransferase center whereas the 2′-*O*-aminoacyl esters are inactive (Chládek *et al.*, 1973, 1974). A similar observation was made independently by Hussain and Ofengand (1973). Also, since there is evidence that the 3′ termini of aminoacyl- or peptidyl-tRNAs are essential for interaction with the peptidyltransferase sites (Rychlík *et al.*, 1967; Monro *et al.*, 1969; Celma *et al.*, 1970), it was concluded that 3′-*O*-aminoacyl-tRNA is used exclusively by peptidyltransferase as an acceptor substrate (Hussain and Ofengand, 1973; Chládek *et al.*, 1973, 1974). The 2′-*O*-aminoacyl-tRNA is formed by the AA-tRNA synthetase reaction (Ofengand and Chen, 1972; Sprinzl and Cramer, 1973; Ofengand *et al.*, 1974) and thus 2′ → 3′ transacylation must occur after enzymic aminoacylation but before peptide bond formation. As a part of our study of the involvement of the 3′ terminus of AA-tRNA in the various stages of protein biosynthesis, we were interested to learn if such migration is a part of the ribosomal mechanism, and it is thus necessary to determine whether or not the 3′ terminus of 2′-*O*-aminoacyl-tRNA can be recognized by the peptidyltransferase A site. In this paper we report that the acceptor terminus of 2′-*O*-aminoacyl-tRNA can indeed be recognized by the acceptor site of peptidyltransferase. A preliminary report of part of this work has appeared (Ringer and Chládek, 1974a).

Materials and Methods

Ribosomes were prepared from late log phase *Escherichia coli* MRE 600 (RNase 1⁻) cells and were washed three times by ultracentrifugation in 0.5 M NH_4Cl as described previously (Chládek *et al.*, 1974). *N*-Acetyl-[^{14}C]phenylalanyl-tRNA, specific activity of 0.4 nmol of [^{14}C]phenylalanine/mg of tRNA, was prepared as described (Chládek *et al.*, 1974). C-A-C-C-A-[^{14}C]Phe was prepared according to the method of Pestka *et al.* (1970) by T_1 RNase digestion of [^{14}C]Phe-tRNA, specific activity of 0.4 nmol of [^{14}C]phenylalanine/mg of tRNA. The chemical synthesis of 2′- and 3′-*O*-aminoacyl nucleoside and 2′- and 3′-*O*-aminoacyl dinucleoside phosphates was described (Chládek *et al.*, 1974; Chládek and Žemlička, 1974). C-A(2′Gly)H (IIb) was also synthesized by general methods (Chládek *et al.*, 1974) as follows.

2′-O-(N-Benzyloxycarbonylglycyl)-3′-deoxyadenosine. The title compound was prepared in 52% yield from 5′-*O*-*p*-methoxytritylcordycepin by a method analogous to that for the *N*-benzyloxycarbonyl-L-phenylalanyl derivative (Chládek *et al.*, 1974). A solid compound was obtained by MeOH–$CHCl_3$ trituration. The product was chromatographically uniform (tlc; silica gel; $CHCl_3$–MeOH, 9:1): mp 171–175°; ultraviolet (uv) (95% EtOH) λ_{max} (nm) 262 (ϵ 14,700); nuclear magnetic resonance (nmr) (CD_3SOCD_3), sodium 2,2-dimethyl-2-silapentane-5-sulfonate (external standard) 8.32 (H_8, s, 1), 8.15 (H_2, s, 1), 7.34 (phenyl s, 5), 7.25 (NH_2, s, 2), 6.10 ($H_{1'}$, d, $J_{1',2'}$ = 3 Hz, 1), 5.05 (CH_2 of benzyloxycarbonyl, s, 2). *Anal.* Calcd for $C_{20}H_{22}N_6O_5 \cdot 1H_2O$ (460.44): C, 52.17; H, 5.25; N, 18.25. Found: C, 52.49; H, 4.96; N, 18.43.

Cytidylyl(3′–5′)-2′-O-glycyl-3′-deoxyadenosine (IIb). The title compound was prepared by condensation of 2′-*O*-(*N*-benzyloxycarbonylglycyl)-3′-deoxyadenosine with N^4-dimethylaminomethylene-2′,5′-di-*O*-tetrahydropyranylcytidine 3′-phosphate, followed by deblocking of the fully protected intermediate as described (Chládek *et al.*, 1974). The yield of the *N*-benzyloxycarbonyl derivative of C-A(2′Gly)H (IIb) was 11% and this intermediate was characterized by the usual methods (Chládek *et al.*, 1974). The title compound was obtained in 68% yield by hydrogenolysis (Chládek *et al.*, 1974) and was characterized by tlc, paper chromatography, electrophoresis, positive reaction with ninhydrin spray, and alkaline hydrolysis to C-3′-dA and glycine. Degradation by pancreatic ribonuclease gave 9% of the 2′ → 5′ isomer and Cp/3′-dA = 0.76; uv (0.01 N HCl) λ_{max} (nm) 267; λ_{min} (nm) 231; 250/260 = 0.72; 280/260 = 0.77; 290/260 = 0.48.

Assay of Poly(U)-Directed Polyphenylalanine Synthesis. Poly(U)-directed polyphenylalanine synthesis was assayed by a modified method of Wood and Berg (1962). Each reaction mixture contained in 0.18 ml: 0.033 M Tris-HCl (pH 7.4), 0.125 M KCl, 0.008 M $MgCl_2$, 0.001 M ATP, 0.0002 M GTP, 0.002 M glutathione, 0.1 mg of tRNA, 100 μM 20 amino acids including [^{14}C]phenylalanine (specific activity equal to 10 Ci/mol), 0.01 mg of poly(U), 0.02 ml of crude S-150 supernatant containing all necessary synthesizing enzymes, and varying amounts of inhibitor compounds as described below (Table I). Reactions were initiated by the addition of 4.0 A_{260} units of ribosomes and incubated for 30 min at 37°. Reactions were terminat-

Table I: Inhibition of Poly(U)-Directed Polyphenylalanine Synthesis by 2′- and 3′-*O*-L-Phenylalanyl Dinucleoside Phosphates.[a]

Inhibitor	% Inhibition at Concn of Inhibitor (M)		
	1.0×10^{-4}	5.0×10^{-5}	1.0×10^{-5}
C-A-Phe	86	64	11
C-A(2′H)Phe	66	34	3
C-A(2′Phe)H	41	13	1
C-A(2′Me)Phe		89	39
C-A(2′Phe)Me		88	30

[a] The assay for the inhibition of polyphenylalanine synthesis by 2′- and 3′-*O*-L-phenylalanyl dinucleoside phosphates was performed by addition of the inhibitors prior to addition of ribosomes. Amino acid incorporation in the absence of inhibitors was 0.5 nmol of [^{14}C]phenylalanine/mg of ribosome per hr. Per cent inhibition is the number of trichloroacetic acid precipitated polyphenylalanine counts in the presence of inhibitor compound relative to the number of CCl_3COOH precipitated counts in the absence of inhibitor. Details of reaction conditions are given under Materials and Methods.

[1] Abbreviations used are: Ac-Phe-tRNA, *N*-acetyl-L-phenylalanyl transfer ribonucleic acid; Ac-Phe, *N*-acetyl-L-phenylalanine; A-Phe, 2′(3′)-*O*-L-phenylalanyladenosine; A(2′Me)Phe, 2′-*O*-methyl-3′-*O*-L-phenylalanyladenosine; A(2′H)Phe, 2′-deoxy-3′-*O*-L-phenylalanyladenosine; A(2′Phe)Me, 3′-*O*-methyl-2′-*O*-L-phenylalanyladenosine; A(2′Phe)H, 3′-deoxy-2′-*O*-L-phenylalanyladenosine (analogous abbreviations are used for the dinucleotide derivatives); tRNA-C-C-3′-dA, tRNA with 3′-deoxyadenosine incorporated at the 3′ end; AA-tRNA, aminoacyl transfer ribonucleic acid.

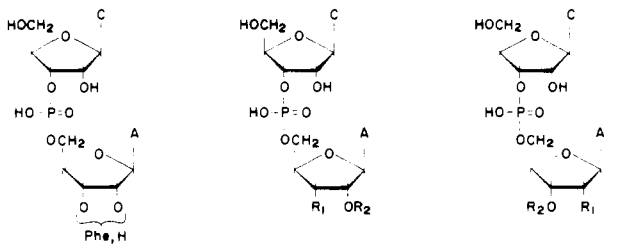
ed by chilling, a 0.1-ml aliquot of each reaction mixture was pipeted onto a Whatman No. 3MM filter paper disk, and the amount of trichloroacetic acid precipitable material was determined as described by Bollam (1968). The degree of inhibition obtained is expressed as the percentage of trichloroacetic acid precipitated counts lost in the presence of inhibitor compounds relative to counts precipitated in inhibitor free controls.

Inhibition of Ac-[^{14}C]Phe-Puromycin Formation. The inhibition of Ac[^{14}C]Phe-puromycin formation from Ac-[^{14}C]Phe-tRNA and puromycin on 70S ribosomes by 2′- and 3′-*O*-L-aminoacyl dinucleoside phosphates and 2′- and 3′-*O*-L-aminoacyl nucleosides was performed as reported previously (Ringer and Chládek, 1974a). A typical reaction mixture of 0.1 ml contained: 0.05 M Tris-HCl (pH 7.4), 0.10 M NH_4Cl, 0.01 M $MgCl_2$, 10 μg of poly(U), 0.1 A_{260} unit of *N*-acetyl-[^{14}C]phenylalanyl-tRNA, either 1.0×10^{-4} or 1.0×10^{-5} M puromycin, and inhibitor compounds I–III at concentrations indicated in the figures. Reactions were initiated by the addition of 4.0 A_{260} units of ribosomes and then incubated for 30 min at 37°. Reaction mixtures in which 2′- and 3′-*O*-L-aminoacyl dinucleoside phosphates had been used as inhibitors were terminated by the addition of 0.1 ml of 0.1 M $Be(NO_3)_2$ and 0.3 M NaOAc (pH 5.5) saturated with $MgSO_4$, and Ac-[^{14}C]Phe-puromycin was extracted with 1.5 ml of ethyl acetate, as described by Monro *et al.* (1968). The radioactivity of 1.0-ml aliquots of the ethyl acetate extract was measured in 10 ml of a 4.5 g of 2,5-diphenyloxazole/100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene/0.25 l. of 2-methoxyethanol/l. of toluene scintillation mixture. Reactions in which 2′- and 3′-*O*-L-aminoacyl nucleosides had been used as inhibitors were terminated and ethyl acetate extraction performed by a procedure modified from the method of Hussain and Ofengand (1972). These reactions were terminated with 0.1 ml of 0.4 N NaOH and the mixture incubated at 37° for 30 min to saponify any ester bonds. Two volumes of saturated $MgSO_4$ were then added, and the assay of Ac-[^{14}C]Phe-puromycin formation was performed as described above. The degree of inhibition is reported as per cent inhibition, calculated as the difference in ethyl acetate extracted counts between inhibitor-free control reactions and reactions to which the inhibitors were added, divided by the counts in inhibitor-free control reactions.

Inhibition of C-A-C-C-A-[^{14}C]Phe Binding to Ribosomes. The binding of C-A-C-C-A-[^{14}C]Phe to ribosomes in the presence of deacylated tRNA was performed basically as described by Hishizawa and Pestka (1971). Inhibition of C-A-C-C-A-[^{14}C]Phe binding to ribosomes by 2′- and 3′-*O*-L-phenylalanyl dinucleoside phosphates was measured in the following mixture (0.1 ml final reaction volume including ribosomes): 0.05 M Tris-HCl (pH 7.4), 0.40 M KCl, 0.04 M $MgCl_2$, 50 μg of tRNA, 1.3 pmol of C-A-C-C-A-[^{14}C]Phe, and inhibitor compounds at the concentrations given (see Figure 4). Reaction mixtures were initiated by addition of 4.6 A_{260} units of ribosomes, incubated for 30 min at 24°, then terminated by the addition of 3 ml of cold buffer containing 0.05 M Tris-HCl (pH 7.4), 0.40 M KCl, and 0.04 M $MgCl_2$. The ribosome · C-A-C-C-A-[^{14}C]Phe complex was isolated by filtration through HAWP-Millipore membranes which were washed with buffer (3 × 3 ml) and dried, and the amount of radioactivity was determined by liquid scintillation counting as described for polyphenylalanine synthesis. The inhibition of C-A-C-C-A-[^{14}C]Phe binding was determined as the difference in the ribosome · C-A-C-C-A-[^{14}C]Phe complex retained on the Millipore membrane in the absence of inhibitor compound and that retained in the presence of inhibitor. This difference was then expressed as a percentage of the radioactivity bound to the ribosomes in the absence of inhibitor.

Results

Poly(uridylic acid) directed synthesis of polyphenylalanine has been routinely used as a cell-free protein synthesizing system to measure general ribosomal function. We have used this system to determine the effect of 2′- and 3′-*O*-L-phenylalanyl dinucleoside phosphates (II and III) on the ribosomal incorporation of free phenylalanine into polyphenylalanine (results in Table I). As can be seen, both 2′- and 3′-*O*-L-phenylalanyl dinucleoside phosphates are effective inhibitors of polyphenylalanine synthesis. Relative to C-A-Phe (I), an analog of the 3′ terminus of Phe-tRNA, C-A(2′Me)Phe (IIIc) and C-A(2′Phe)Me (IIc) were better



inhibitors of ribosomal phenylalanine incorporation while C-A(2′H)Phe (IIIa) and C-A(2′Phe)H (IIa) appeared to be somewhat weaker inhibitors than C-A-Phe (I).

The peptidyltransferase activity of these 2′- and 3′-*O*-L-phenylalanyl dinucleoside phosphates has been previously investigated (Chládek *et al.*, 1974), and it was found that while C-A-Phe (I), C-A(2′Me)Phe (IIIc), and C-A(2′H)Phe (IIIa) were active as peptidyltransferase acceptors, C-A(2′Phe)Me (IIc) and C-A(2′Phe)H (IIa) were inactive. In addition, both the 2′- and 3′-*O*-glycyl derivatives, C-A(2′H)Gly (IIIb) and C-A(2′Gly)H (IIb), show only slight acceptor activity (Ringer and Chládek, 1974b, and unpublished results). While the inhibitory ability of C-A-Phe (I), C-A(2′H)Phe (IIIa), and C-A(2′Me)Phe (IIIc) in polyphenylalanine synthesis can be at least partially attributed to their puromycin-like acceptor activity, the ability of 2′-*O*-aminoacyl esters II to act as inhibitors had to be further investigated in less complex systems.

In order to further elucidate the nature of the inhibitory effect of 2′-*O*-aminoacyl dinucleoside phosphates (II) on protein biosynthesis, their ability to influence ribosomal peptidyltransferase-mediated Ac-[^{14}C]Phe-puromycin formation in the Ac-Phe-tRNA · poly(U) · 70S ribosome complex was examined.

Figure 1a,b and Table II-A indicate that both 2′- and 3′-nonisomerizable aminoacyl esters II and III are inhibitors of Ac-[^{14}C]Phe-puromycin formation (1.0×10^{-4} M puromycin). As can be seen from Table II-A, 2′-*O*-aminoacyl derivatives always displayed higher inhibitory activities than their 3′ counterparts III. The activities of the 2′-*O*-aminoacyl esters II are comparable to that of C-A-Phe (I) which can exist as either a 2′ or 3′ isomer. The different shape of the C-A-Phe (I) curve may imply that both C-A-Phe (I) and puromycin react with Ac-[^{14}C]Phe-tRNA, C-

Table II: Summary of Data of Figures 1, 2, and 4 Showing Concentrations of Aminoacyl Oligonucleotides I–III at Which 50% Inhibition Occurs in the Systems A, Ac-[^{14}C]-Phe-Puromycin Formation at 10^{-4} M Puromycin; B, Ac-[^{14}C]Phe-Puromycin Formation at 10^{-5} M Puromycin; and C, C-A-C-C-A-[^{14}C]Phe Ribosomal Binding.

Compound	A	B	C
C-A-Phe, I	2.5×10^{-5}		2.5×10^{-4}
C-A(2′Phe)H, IIa	4.2×10^{-5}	3.4×10^{-5}	2.5×10^{-4}
C-A(2′Gly)H, IIb	5.6×10^{-5}	4.0×10^{-5}	
C-A(2′Phe)Me, IIc	3.3×10^{-5}[a]		8.3×10^{-5}
C-A(2′H)Phe, IIIa	1.2×10^{-4}	3.5×10^{-6}	3.2×10^{-4}
C-A(2′H)Gly, IIIb	2.5×10^{-4}	2.2×10^{-4}	
C-A(2′Me)Phe, IIIc	7.5×10^{-5}[a]		4.6×10^{-5}

[a] Data from Ringer and Chládek (1974a).

A-Phe (I) being an excellent acceptor (Chládek *et al.*, 1974).

Inhibition experiments with aminoacyl dinucleoside phosphates IIa, IIb, IIIa, and IIIb at 10^{-5} M concentration of puromycin were also performed (Figure 2 and Table II-B) and the results appear to agree with the general trend observed at 10^{-4} M puromycin concentration. Both C-A(2′Phe)H (IIa) and C-A(2′Gly)H (IIb) displayed roughly equal activity. The curve for C-A(2′H)Phe (IIIa) reflects its significant ability to compete with puromycin for an available donor (Ringer and Chládek, 1974b). A similar shape of the curve was observed for C-A-Phe (I) at 10^{-4} M puromycin concentration (Figure 1a).

In order to examine the nature of the inhibitory effect of C-A(2′Phe)H (IIa) on Ac-Phe-puromycin formation, a kinetic analysis was performed under conditions similar to those used by Pestka (1970). A Lineweaver–Burk plot (Figure 3) shows that C-A(2′Phe)H does not demonstrate simple competitive inhibition with puromycin. Instead, a more complex inhibition resulting in mixed kinetics is observed.

To further investigate the interaction of 2′- and 3′-*O*-L-phenylalanyl dinucleoside phosphates with the peptidyltransferase A site, their ability to inhibit C-A-C-C-A-Phe binding to ribosomes was investigated. The binding system employed deacylated tRNA, which has been previously shown to stimulate the binding of C-A-C-C-A-Phe to both 70S ribosomes (Pestka, 1969) and 50S subunits (Hishiazwa and Pestka, 1971). The deacylated tRNA should also direct binding of C-A-C-C-A-Phe to the peptidyltransferase A site (Watanabe, 1972).

Binding of C-A-C-C-A-[^{14}C]Phe to 70S ribosomes is inhibited by chloramphenicol and sparsomycin (at antibiotic concentrations of 10^{-4} M the observed inhibitions of C-A-C-C-A-[^{14}C]Phe binding to ribosomes were 68 and 75%, respectively), and these levels of inhibition are in agreement with previous reports on the 50S system (Hishizawa and Pestka, 1971). A check for formation of [^{14}C]Phe-puromycin in the presence of 1.0×10^{-3} M puromycin was also conducted in an effort to detect the presence of reactable C-A-C-C-A-[^{14}C]Phe bound to the P site. Ethyl acetate extraction of the alkalized reaction mixture (Pestka, 1970) showed that no [^{14}C]Phe-puromycin formation had occurred under binding-reaction conditions.

Figure 4a,b and Table II show that in addition to C-A-Phe (I), the nonisomerizable 2′- and 3′-*O*-L-phenylalanyl dinucleoside phosphates (IIa, IIc, IIIa, and IIIc) were able

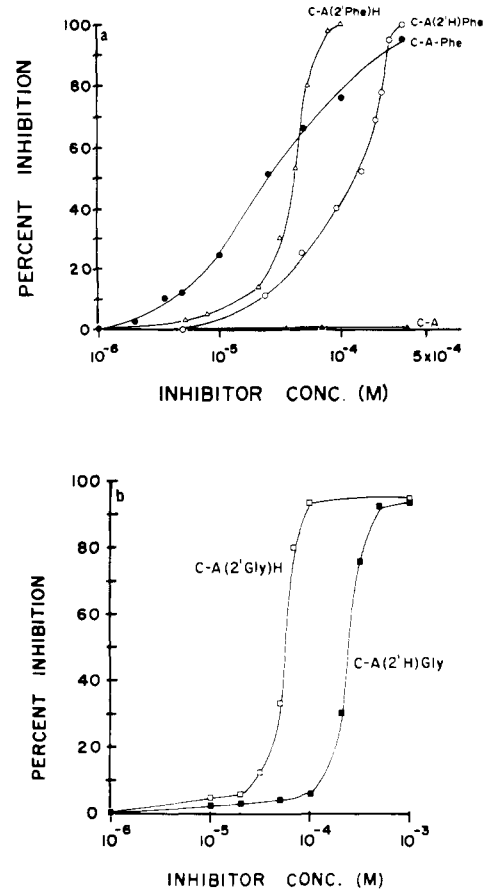


FIGURE 1: The inhibition of Ac-Phe-puromycin formation in the presence of 2′- and 3′-*O*-aminoacyl dinucleoside phosphates. Compounds I–III were present at concentrations indicated on the abscissa and puromycin concentration was 1.0×10^{-4} M. Ribosomes were added last to initiate the reactions. Details of the reaction procedure and conditions are given in the Materials and Methods section. Per cent inhibition represents the difference in ethyl acetate extracted Ac-[^{14}C]Phe-puromycin counts in the presence and in the absence of inhibitor, relative to the amount of Ac-[^{14}C]Phe-puromycin formed without inhibitor present. Ac[^{14}C]Phe-puromycin (100%) formed was equal to 1650 cpm: (a) (Δ) C-A(2′Phe)H (IIa); (○) C-A(2′H)Phe (IIIa); (●) C-A-Phe (I) (replotted from Ringer and Chládek, 1974a); (b) (□) C-A(2′Gly)H (IIb); (■) C-A(2′H)Gly (IIIb).

to inhibit C-A-C-C-A-[^{14}C]Phe binding to the ribosomes. C-A-Phe (I) C-A(2′Phe)H (IIa), and C-A(2′H)Phe (IIIa) had similar inhibitory activities, with maximum inhibitions of 75%, whereas C-A(2′Me)Phe (IIIc) and C-A(2′Phe)Me (IIc) demonstrated stronger affinity for the ribosomal A site.

Studies of the inhibition of Ac-Phe-puromycin formation by the 2′- and 3′-*O*-L-aminoacyl nucleoside derivatives, A-Phe, A(2′Phe)H, A(2′H)Phe, A(2′Phe)Me, and A(2′Me)Phe, were conducted at 1.0×10^{-5} M puromycin concentration in order to allow for more sensitive measurement of inhibition by the weakly bound aminoacyl nucleosides. As can be seen in Figure 5, all nucleosides tested were inhibitors of Ac-Phe-puromycin formation. The order of their inhibitory activities was similar to the order of their acceptor activities in the peptidyltransferase reaction (acceptor activities in decreasing order: A-Phe, A(2′Me)Phe, A(2′H)Phe, A(2′Phe)Me, and A(2′Phe)H; Chládek *et al.*, 1974). It is surprising that A(2′H)Phe is a good inhibitor, even though it is known to be a very poor acceptor (Rychlík *et al.*, 1969). At the present time it is difficult to understand the biological significance of this finding, considering the

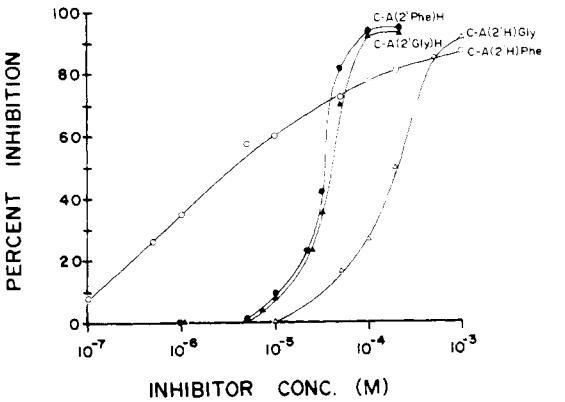


FIGURE 2: The inhibition of Ac-Phe-puromycin formation in the presence of C-A(2′Phe)H (IIa), C-A(2′Gly)H (IIb), C-A(2′H)Phe (IIIa), and C-A(2′H)Gly (IIIb). Reactions were performed as in Figure 1 except that the puromycin concentration was 1.0×10^{-5} M. Per cent inhibition represents the difference in the amount of Ac-Phe-puromycin formed in the presence and absence of inhibitor compound: (○) C-A(2′H)Phe (IIIa); (●) C-A(2′Phe)H (IIa); (△) C-A(2′H)Gly (IIIb); (▲) C-A(2′Gly)H (IIb).

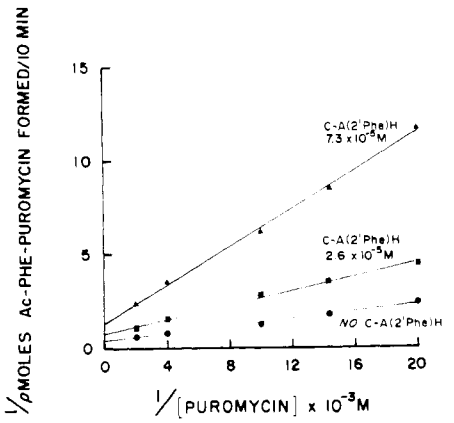


FIGURE 3: Lineweaver–Burk plot of the effect of C-A(2′Phe)H (IIa) on the rate of Ac-[^{14}C]Phe-puromycin formation as a function of puromycin concentration. The reaction conditions for measuring the inhibition of Ac-[^{14}C]Phe-puromycin formation were those described under Materials and Methods with the exceptions that the puromycin concentration was varied as indicated on the abscissa (5.0×10^{-6} to 5.0×10^{-5} M) and C-A(2′Phe)H when present was at either 2.6×10^{-5} or 7.3×10^{-5} M. It was ascertained that the formation of Ac-[^{14}C]Phe-puromycin in the presence of 1.0×10^{-4} M puromycin was linear for 12 min at 30°. Reactions were incubated for 10 min at 30°: (●) no C-A(2′Phe)H; (■) 2.6×10^{-3} M C-A(2′Phe)H; (▲) 7.3×10^{-5} M C-A(2′Phe)H.

fact that extremely high concentrations of nucleoside inhibitors had to be used in order to achieve any measurable inhibition. The 2′- and 3′-*O*-L-phenylalanyl nucleosides A(Phe), A(2′Phe)H, A(2′Phe)Me, A(2′H)Phe, and A(2′Me)Phe were also examined as inhibitors of C-A-C-C-A-[^{14}C]Phe binding, and they showed little or no inhibition of binding (data not shown).

Discussion

We found that both 2′ and 3′-nonisomerizable aminoacyl derivatives of dinucleoside phosphates IIIa, IIIc, IIa, and IIc inhibit poly(U) dependent incorporation of phenylalanine into polypeptide, although only the 3′ esters IIIa and IIIc are acceptors of the peptide chain in the peptidyltransferase reaction (Chládek *et al.*, 1974). In principle, inhibitors having a structural resemblance to the acceptor terminus of AA-tRNA may function in several distinct steps of protein biosynthesis, *e.g.*, enzymic charging of tRNA, binding to the A site of ribosomes, and the peptidyltransferase reaction. Thus, more simplified systems were employed in order to determine the precise target of the particular inhibitor.

In the first system, transfer of the Ac-Phe residue from the Ac-Phe-tRNA · poly(U) · 70S ribosome complex to puromycin is strongly inhibited by both the 2′- and 3′-*O*-aminoacyl esters IIa, IIb, IIc, IIIa, and IIIc (see also Ringer and Chládek, 1974a), whereas the 3′-*O*-glycyl derivative C-A(2′H)Gly (IIIb) is less effective. Since the 3′-*O*-aminoacyl oligonucleotides III can act as acceptors (Chládek *et al.*, 1974), and since increasing the concentration of puromycin overcomes the inhibitory effect of the 2′ esters C-A(2′Phe)H (IIa) and C-A(2′Phe)Me (IIc), we assume that both the 2′- and 3′-*O*-aminoacyl oligonucleotides II and III act at the A site of peptidyltransferase (Ringer and Chládek, 1974a).

From a comparison of the inhibitory activities of 2′- and 3′-*O*-glycyl derivatives IIb and IIIb, it is clear that the 2′ ester C-A(2′Gly)H (IIb) is a better inhibitor than its 3′ counterpart C-A(2′H)Gly (IIIb). Since both compounds are only weak acceptors in the peptidyltransferase reaction (Ringer and Chládek, 1974b, and unpublished results), their inhibitory properties largely reflect their binding affinities to the peptidyltransferase A site, indicating a greater binding affinity for the 2′ isomer C-A(2′Gly)H (IIb). Although a direct comparison of the inhibitory activities of 2′- and 3′-*O*-L-phenylalanyl derivatives IIa and IIIa is complicated by the significant acceptor activity of the 3′ isomer IIIa, especially at low (10^{-5} M) puromycin concentration, the same general trend is observed.

Comparison of the inhibitory activities of the 3′-*O*-aminoacyl esters C-A(2′H)Phe (IIIa) and C-A(2′H)Gly (IIIb) shows that the L-phenylalanyl derivative IIIa is a better inhibitor than the glycyl derivative IIIb. This may be expected in light of the fact that 2′(3′)-*O*-aminoacyladenosines with aromatic amino acids are better acceptors in the peptidyltransferase reaction (undoubtedly acting as 3′ isomers) than analogous derivatives with aliphatic amino acids, with the glycyl derivatives being the least active (Rychlík *et al.*, 1970a, and references therein). Surprisingly, no such trend was observed with the 2′-*O*-aminoacyl esters C-A(2′Phe)H (IIa) and C-A(2′Gly)H (IIb); both IIa and IIb displayed roughly the same activity. This finding could mean that the aminoacyl portions of 2′- and 3′-*O*-aminoacyl oligonucleotides II and III act at spatially separated, mutually exclusive loci of the peptidyltransferase A site ("2′- and 3′-*O*-aminoacyl loci"). The mixed type of kinetics obtained for the inhibition of Ac-Phe-puromycin formation by C-A(2′Phe)H (IIa) seems to support this interpretation. These results are strikingly similar to the results of Goldberg and Mitsugi (1967), who have observed mixed kinetics for the inhibition of Ac-Phe-puromycin formation by chloramphenicol, another peptidyltransferase A site inhibitor (Nierhaus and Nierhaus, 1973). Goldberg and Mitsugi (1967) interpreted these findings to be a consequence of allosteric inhibition (see also Celma *et al.*, 1970; Lessard and Pestka, 1972).

In order to obtain proof that the 2′-*O*-esters IIa and IIc interfere with the binding function of the peptidyltransferase A site, we investigated the effect of the inhibitors IIa, IIc, IIIa, and IIIc on the binding of C-A-C-C-A-Phe to 70S ribosomes (Pestka, 1969). The binding of C-A-C-C-A-Phe to the A site of peptidyltransferase is directed by an excess

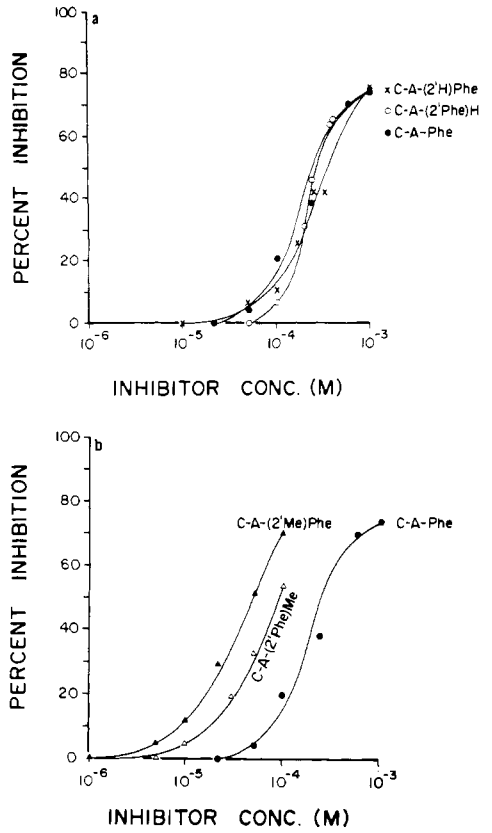


FIGURE 4: Inhibition of C-A-C-C-A-[^{14}C]Phe binding to ribosomes by 2′- and 3′-*O*-L-phenylalanyl dinucleoside phosphates. Details of the assay conditions are given under Materials and Methods. Per cent inhibition is the difference in ribosome · C-A-C-C-A-[^{14}C]Phe complex retained on the Millipore membrane in the absence and in the presence of inhibitor, relative to the amount bound in the absence of inhibitor. Zero per cent inhibition equals 0.6 pmol of C-A-C-C-A-[^{14}C]Phe bound to ribosomes: (a) (×) C-A(2′H)Phe (IIIa); (○) C-A(2′Phe)H (IIa); (●) C-A-Phe (I); (b) (▲) C-A(2′Me)Phe (IIIc); (△) C-A(2′-Phe)Me (IIc); (●) C-A-Phe (I).

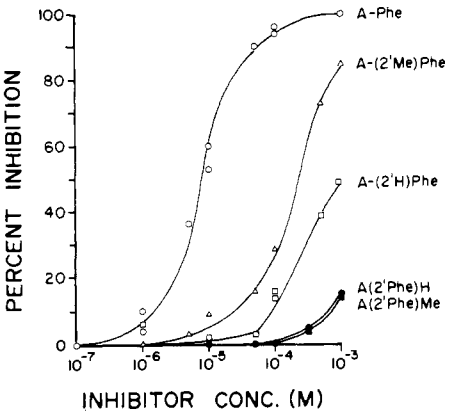


FIGURE 5. The inhibition of Ac-Phe puromycin formation in the presence of 2′- and 3′-*O*-aminoacyl nucleosides. Compounds A-Phe, A(2′Me)Phe, A(2′H)Phe, A(2′Phe)H, and A(2′Phe)Me were assayed for inhibitory activity at the concentrations indicated in the figure. Reaction conditions were the same as those described in Figure 1 except that the puromycin concentration was lowered to 1.0×10^{-5} M. Per cent inhibition refers to the difference in the amount of Ac-Phe-puromycin formed in the presence and absence of the inhibitor compound: (○) A-Phe; (△) A(2′Me)Phe; (□) A(2′H)Phe; (●) A(2′Phe)H; and (■) A(2′Phe)Me.

of deacylated tRNA (Pestka, 1969; Watanabe, 1972). As can be seen from the results, the binding of C-A-C-C-A-Phe is strongly inhibited by the nonisomerizable models IIa, IIc, IIIa, and IIIc as well as by C-A-Phe (I).[2]

Unlike the marked differences in activities between the 2′ and 3′ isomers observed in the inhibition of Ac-Phe-puromycin formation, there are comparatively small differences in inhibition of C-A-C-C-A-Phe binding. This possibly reflects the greater binding affinity of C-A-C-C-A-Phe for the ribosome compared to that of puromycin. Another possibility, that C-A-C-C-A-Phe may bind to ribosomes as either the 2′ or 3′ isomer, cannot as yet be ruled out. In that case, differences in the inhibitory effects of the 2′ or 3′ isomers might be obscured. The system employing puromycin may therefore be more sensitive to the differences in the affinities of the 2′ and 3′ isomers.

We conclude that the 2′-*O*-aminoacyl oligonucleotides with the terminal sequence of tRNA are bound to the peptidyltransferase A site. On the basis of the structural resemblance of compounds II to the acceptor end of 2′-*O*-aminoacyl-tRNA, we suggest that the same may hold true for the 3′ terminus of 2′-*O*-aminoacyl-tRNA.

Although the final verdict has to await direct evidence of the binding of 2′- and 3′-*O*-aminoacyl oligonucleotides to the peptidyltransferase A site, it seems reasonable to assume that the acceptor terminus of 2′-*O*-aminoacyl-tRNA may be bound to the peptidyltransferase A site at least as tightly as the acceptor terminus of 3′-*O*-aminoacyl-tRNA. Furthermore, as was recently found by Chinali *et al.* (1974; personal communication of A Parmeggiani), the Phe-tRNA-C-C-3′-dA (2′ ester) can be bound to ribosomes in the presence of the elongation factor EF-T_u and GTP.

On this basis, we are tempted to suggest that 2′-*O*-aminoacyl-tRNA may be bound to the ribosomal A site in the step preceding peptide bond formation. The binding of the aminoacyl portion of 2′-*O*-aminoacyl-tRNA might occur at the 2′-*O*-aminoacyl locus of the peptidyltransferase A site (*vide supra*). The "correct" 3′ isomer for the subsequent peptide forming step is then supplied by 2′ → 3′ transacylation which occurs at the peptidyltransferase A site. The transacylation step might be concerted with GTP hydrolysis, which is known to occur during enzymic binding of AA-tRNA (Lipmann, 1969).

It was observed by Pestka (1967) that the ester bond of AA-tRNA is considerably stabilized toward hydrolysis while AA-tRNA is bound to ribosomes. Since it is well known that activation of this ester bond is partly due to hydrogen bonding from the neighboring hydroxyl group (Bruice and Fife, 1962; Rammler and Khorana, 1963; Chládek *et al.*, 1970), it may be assumed that this hydroxyl group is bound to the peptidyltransferase A site locus and the particular isomer of AA-tRNA is thus prevented from migration or hydrolysis (see also Rychlík *et al.*, 1969). It then follows that an "isomerase"[3] enzymic activity of the peptidyltransferase A site may be necessary to catalyze the required 2′ → 3′ transacylation even though such acyl migration is extremely fast in solution.

Added in Proof

After this paper was submitted, the report of Chinali *et al.* (1974) appeared showing that Phe-tRNA-3′dA (2′

[2] Inhibition of C-A-C-C-A-Phe binding to 70S ribosomes by puromycin in the absence of deacylated tRNA has been shown by Lessard and Pestka (1972).

[3] The existence of a specific 2′ → 3′ or 3′ → 2′ isomerase in cells was first postulated by Wolfenden *et al.* (1964).

ester) can function as a peptidyltransferase acceptor substrate, albeit at a much slower rate than Phe-tRNA. While this finding supports our contention that the 3′ terminus of 2′-AA-tRNA can be accommodated by the A site of peptidyltransferase, it is in conflict with previous suggestions that only 3′-AA-tRNA acts as an acceptor in the peptidyltransferase reaction.

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