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Studies on the reactivity of ribosome-bound peptidyl transferase for various modified substrates

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STUDIES ON THE REACTIVITY OF RIBOSOME-BOUND PEPTIDYL TRANSFERASE FOR VARIOUS MODIFIED SUBSTRATES

by

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ABBREVIATIONS

(In alphabetical order)

A_{260} unit	One A_{260} unit is the amount of material which in 0.1 ml would yield a value of 1.0 for the absorbance measured at 260 nm in a cuvette with a path length of 1.0 cm.
ACCA(Tyr)	Tyrosyl-adenyl-cytidyl-cytidyl-adenosine
A(Tyr) or Ado(Tyr)	Tyrosyl-adenosine
A(Phe), Ado(Phe)	Phenylalanyl-adenosine
Ado^{ox-red}(Phe)	Oxidized-reduced phenylalanyl-adenosine
AA-tRNA	aminoacyl-tRNA
A(Phe-Phe)	2'(3')-O-(L-phenylalanyl-L-phenylalanyl) adenosine
ATP	adenosine-5'-triphosphate
ADP	adenosine-5'-diphosphate
AMP	adenosine - 5'-monophosphate
A(Gly)	2'(3')-O-glycyl-adenosine
A(Lys)	2'(3')-O-glycyl-adenosine
A(Tyr)	2'(3')-O-tyrosyl-adenosine
A(Phe)	2'(3')-O-phenylalanyl-adenosine
BD-cellulose	benzoylated DEAE-cellulose
BrAcphetRNA	bromoacetyl-phenylalanyl-tRNA
^{o} C	degrees centigrade
cm	centimeter
CMP	cytidine-5'-monophosphate
[^{14}C] or [^{3}H]Phe-tRNA	tRNA acylated with [^{14}C] or [^{3}H]phenylalanine (and analogously [^{14}C]Tyr-tRNA)
CM-cellulose	carboxymethyl-cellulose

C(Phe)	2'(3')-O-phenylalanyl-cytidine
-CCA	adenyl-cytidylyl-cytidylyl
CpPAN-Gly	5'-O-cytidyl-3'-N-glycyl-puromycin aminonucleoside
CACCA-(Ac-L-Leu)	2'(3')-O-(N-acetyl-L-leucyl) ester of the pentanucleotide $C_pA_pC_pC_pA$
CACCA(Phe)	The 3'-terminal pentanucleotide fragment of Phe-tRNA^{phe}
CACCA^{ox-red}(Phe)	phenylalanyl-pentanucleotide in which the 3'-terminal adenosine was oxidized-reduced
DCC	N,N'-dicyclohexylcarbodiimide
DTT	Dithiothreitol
DEAE	diethylaminoethyl
dA(Phe)	3'-O-phenylalanyl-2'-deoxyadenosine
E. coli	Escherichia coli
EDTA	ethylenediamine tetra acetic acid
fMet-tRNA	2'(3')-O-(N-formyl-L-methionyl)-tRNA
GMP	Guanosine-5'-monophosphate
GDP	Guanosine-5'-diphosphate
GTP	Guanosine-5'-triphosphate
G(Phe)	2'(3')-O-phenylalanyl-guanosine
IF-1, IF-2, IF-3	Initiation factors, 1, 2, 3
$(I_p)_5I$	Hexainosinic acid
im-benzyl-Hist	imidazole-benzyl-L-histidine
KOAc	Potassium acetate
$Mg(OAc)_2$	magnesium acetate
$MgSO_4$	magnesium sulfate
methyl-pA(Phe)	5'-O-(methyl-phosphoryl)-(2'(3')-O-L-phenylalanyl)(adenosine)
nm	nanometer

NAcphetRNA	N-acetyl-phenylalanyl-tRNA
NaCl	sodium chloride
NaOAc	sodium acetate
NAcphe	N-acetyl-phenylalanine
NAcphe-phe	N-acetyl-phenylalanylphenylalanine
NAcphe-phe-Ado	2'(3')-O-N-acetylphenylalanylphenylalanyl-adenosine
NAcphe-phe-Ado^{ox-red}	2'-O-N-acetylphenylalanylphenylalanyl-adenosine (oxidized-reduced)
ox-red	oxidized-reduced
oligo I	oligoinosinic acid
[^{32}P] ATP	γ-[^{32}P]labelled ATP
PEP	phosphoenol pyruvate
Pi	inorganic phosphate
PA(Gly)	2'(3')-O-glycyladenosine-5'-monophosphate
PAN	Puromycin aminonucleotide (6-dimethylamino-9(3'-amino,3'deoxyribosyl)purine
PAN-L-phe	3'-N-L-phenylalanyl-puromycin amino-nucleoside
p-PAN-Gly	5'-O-phosphoryl-3'-N-glycyl-puromycin aminonucleoside
PAN-Bzl-L-Cys	S-benzyl-L-Cysteinyl-puromycin amino-nucleoside. Other puromycin analogues are abbreviated similarly.
Phe	L-phenylalanine. All other amino acids not designated as L or D are of L configuration
Phe-tRNA^{ox-red}	tRNA^{ox-red}, acylated with phenylalanine
Phe-tRNA^{cont}	Phe-tRNA which has been subjected to oxidation conditions, deacylated, exposed to reduction conditions and then acylated with phenylalanine.
Phe-tRNA^{unt}	Untreated phe-tRNA; not subjected to any chemical exposure.

Poly U	Polyuridylic acid
Poly AU	Polyadenylic-uridylic acid
Poly I	Polyinosinic acid
R_f	Ratio of the distance travelled by the material to that travelled by the solvent.
T	Peptide chain elongation factor from Escherichia coli.
Tu	One of the peptide chain elongation factors from Escherichia coli.
tRNA	transfer ribonucleic acid
$tRNA^{ox}$, $tRNA^{ox-red}$	tRNA which has been oxidized with periodate, first oxidized with periodate and then reduced with sodium borohydride respectively.
t-BOC	tertiary-butyloxycarbonyl
$TrAdo^{ox}$	trityladenosine (oxidized)
$TrAdo^{ox-red}$	trityl-adenosine (oxidized-reduced)
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)amino methane
Tyr	L-tyrosine
Tyr-oligonucleotide	Tyrosyl-oligonucleotide
Trityl	triphenylmethyl
TrAdo	5'-O-trityl-adenosine
19mer-Tyr	AAUCCUUCCCCCACCACCA(Tyr)
4mer-Tyr	ACCA(Tyr)
UMP	Uridine-5'-monophosphate
U(Phe)	2'(3')-O-phenylalanyl-uridine

CHAPTER I

INTRODUCTION

Peptide chains of proteins are synthesized on the ribosome by a series of cyclic reactions. In each cycle of the reaction a new peptide bond is formed through the transfer of the nascent peptide residue from the CpCpA terminus of a pre-existing tRNA molecule to the amino group of an amino acid residue bound to the CpCpA terminus of an incoming tRNA molecule. The transfer of the nascent peptide chain is catalyzed by a ribosome-bound enzyme, peptidyl transferase (Traut and Monro, 1964; Monro et al., 1967; Rychlik, 1965, 1966), shown to be localized on the 50S ribosomal subunit (Monro et al., 1967). The active site of peptidyl-transferase is composed of two binding sites; the donor or the peptidyl-tRNA (P) site and the acceptor or aminoacyl-tRNA (A) site (Watson, 1964; Monro et al., 1967; Rychlik, 1968a,b,; Rychlik et al., 1969).

1. Mechanism of the Peptidyl Transferase reaction

The mechanism of the transfer reaction involves a nucleophilic attack by the primary α-amino group of the incoming aminoacyl-tRNA bound at the acceptor site of the ribosome on the carboxyl ester linkage between the growing polypeptide chain and its tRNA bound at the ribosomal donor site. This results in the cleavage of the ester link with the subsequent formation of a peptide bond (Allen and Zamecnik, 1962; Nathans, 1964a, 1964b). Brattsten et al., (1965) have suggested the possibility of a

peptidyl-enzyme intermediate during the reaction, but there is no evidence for or against such a proposal as yet.

Peptidyl transferase catalyzes the formation of a peptide bond not only between whole molecules of transfer RNAs but also between terminal fragments of peptidyl and aminoacyl-tRNAs. Peptidyl-tRNA can be replaced by 3'-oligonucleotide fragments from fMet-tRNA or from N-acetyl-aminoacyl-tRNA using puromycin as acceptor (Monro et al., 1968; Monro and Marcker, 1967; Mercer and Symons, 1972) and aminoacyl-tRNA by aminoacyl-adenosine (Rychlik et al., 1970) or its structural analogue, the antibiotic puromycin (Monro et al., 1967; Nathans, 1967; Pestka and Hintikka, 1971; Pestka, 1972 a, 1972 b.). The puromycin reaction has been extensively studied and has provided much information on the mechanism of protein biosynthesis.

2. Molecular mechanism of action of puromycin

The normal acceptor substrate *in vivo* is aminoacyl-tRNA coded for by mRNA; however, both *in vivo* and *in vitro* the antibiotic puromycin can replace this substrate. Puromycin is structurally very similar to and acts as an analogue of the 3'-aminoacyl adenosine portion of aminoacyl-tRNA (Yarmolinsky and de la Haba, 1959). The structural differences between puromycin and A(Tyr) are small. There are three additional methyl groups and a stable 3' amide linkage in puromycin compared to the alkali labile 2'(3')-O-ester linkage in A(Tyr) (see Figure 20C). During protein synthesis puromycin and its analogues presumably binds at least transiently to the A site so that peptidyl transferase can catalyze the attack of the primary α-amino group of puromycin on the ester link of peptidyl-tRNA

leading to the formation of a puromycin-peptide (Allen and Zamenick, 1962; Nathans, 1964a, 1964b). The puromycin-peptide so formed dissociates from the ribosomes, thus prematurely terminating protein synthesis.

3. Effect of cations and pH on peptidyl transferase activity

The effect of cations and pH have been studied, using the puromycin reaction with(a) washed E. coli ribosomes charged with polyphenylalanine (b) terminal fragments from F-Met-tRNA CAACCA(fMet) and CCA(fMet) in the presence of 50S ribosomal subunits (the "fragment reaction") (c) washed E. coli ribosomes charged with polylysine and (d) polyribosome extracts. In all of the above assays the reaction required the presence of suitable divalent (Mg^{++}) and monovalent (K^{+}) cations, although the optimum concentration and response varied with the nature of the assay. In (a), Mg^{++} was replaceable with Ca^{++} and monovalent cations were active in the order of $NH_4^{+} > K^{+} = Rb^{+} > Cs^{+}$ (Traut and Monro, 1964) while in (b) Ca^{++} was inert and $K^{+} > Rb^{+} > NH_4^{+}$ while Cs^{+} was inert (Maden and Monro, 1968). In (d) Mn^{++} can substitute for Mg^{++} but Ca^{++} can only do so very poorly. A single K^{+} activates the monosomes in polyribosome complex (Pestka, 1972a).

The response to pH value was similar in assays (a), (b), and (d). All of them were progressively inhibited below pH 8.5 (Maden and Monro, 1968; Pestka, 1972) and above pH 9.0. No such study has been reported for (c).

The localization of the effect of cations and pH to one or more of the three specific steps of the overall process of peptide bond formation,

namely (i) binding of donor substrate to the P site, (ii) binding of acceptor substrate to the A site and (iii) peptide bond formation, is largely unknown. However, Pestka et al., (1970) have studied the binding of various 2'(3')-O-aminoacyl-oligonucleotides and N-acetyl-phenylalanyl-oligonucleotides to E. coli ribosomes (i.e., (i) and (ii) above), and 50S subunits (Hishizawa and Pestka, 1971; Celma et al., 1970; Cerna, 1971). The monovalent and divalent cation requirements for binding were similar to, and may reflect the overall requirements for, the peptidyl transferase reaction cited above. The effect of pH was not reported.

The effect of cations was proposed to be a general one on ribosome conformation (Maden and Monro, 1968). However, Vogel et al., (1970), Miskin et al., (1970), and Eilman (1970) have shown that the loss of peptidyl transferase activity on removal of monovalent cations from the 50S E. coli ribosomal subunit led to no significant change in the physical properties (melting profile, optical rotatory dispersion, sedimentation properties and content of ribosomal RNA and protein) of the 50S particle. On the other hand, the requirement for incubation at 50° C in the presence of monovalent cations for the regeneration of peptidyl transferase activity from inactive 50S particles suggests that a conformational change was involved. Possibly the effect involves only a small local conformational change at the peptidyl transferase center.

The pH response of peptidyl transferase referred to above differs from that of the overall process of protein synthesis (Matthaei and Nirenberg, 1961). Moreover, there was no comparable effect of pH on the binding of chloramphenicol (Fernandez-Munoz et al., 1968), erythromycin (Mao and Putterman, 1969), lincomycin (Chang and Weisblum, 1967)

or of mRNA binding to the ribosome (Dahlberg and Haselkorn, 1967). Consequently, the effect is unlikely to be a non-specific one on ribosomal conformation. Rather, the results suggested the involvement in the peptidyl transferase reaction of a functional group with a pK_a in the approximate range of pH 7.5-8.0. The same pH effect was obtained when α-hydroxy-puromycin was used as the acceptor substrate, thus eliminating the involvement of the α-amino group of puromycin (pK_a approximately 7.0; Maden and Monro, 1968) in the pH effect (Fahnestock et al., 1970). An alternative possibility is that a specific group of a 50S ribosomal protein, for example, an imidazole residue or an N-terminal amino group might be involved in catalysis of the reaction (Maden and Monro, 1968; Fahnestock et al., 1970; Pestka, 1972a).

4. Attempts to Isolate peptidyl transferase

Peptidyl transferase is an integral part of the 60S mammalian and 50S bacterial ribosomal subunit (Monro et al., 1967; Vazquez et al., 1969a). The subunits are complex. The 50S particle consists of 23S and 5S ribosomal RNA and 34 proteins (Kaltschmidt and Wittmann, 1970) and the 60S particle consists of 28S, 7S and 5S ribosomal RNA and approximately 30 proteins (Pene et al., 1968; Gould, 1970).

Attempts to determine the actual protein responsible for peptidyl transferase activity include the following: (a) Inactivation of peptidyl transferase by the stepwise removal of 50S proteins and regeneration by addition of the essential components and (b) affinity labelling with peptidyl-tRNA analogues and antibiotic analogues.

a. Staehelin et al., (1969) reported that a group of five or six proteins, $SP^{\alpha-\beta}$, derived from the 50S ribosomal subunit of E. coli by salt extraction were not required for peptidyl transferase activity while a second group of five or six proteins, $SP^{\beta-\delta}$, were essential for such activity. The main virtue of this method is in its usefulness in eliminating certain proteins. Using LiCl-treatment, Nierhaus and Montejo (1973) isolated a 50S-derived core (0.8 c) which was inactive, but whose activity could be reconstituted by protein L_{11}. Whether L_{11} displays the enzymatic activity itself, is a part of the enzymatic center, or controls the conformation of the active center, is not known.

Should the site of action of antibiotics such as lincomycin and erythromycin prove to be at the peptidyl transferase stage, then the identification of altered proteins from antibiotic resistant 50S subunits of certain bacterial mutants (Krembel and Apirion, 1968; Otaka et al, 1970) would also offer an alternative approach to the identification of the peptidyl transferase protein.

b. Affinity labelling technique has recently been used in a number of laboratories using various derivatized AA-tRNA and derivatized antibiotics. In these cases the affinity reagent attaches covalently with reactive groups in proteins (or RNA) on the ribosome. The rationale for these experiments is as follows.

It is known that the formation of peptide bond is localized exclusively on the 50S particle (Nomura, 1970; Monro, 1967). Therefore, the protein (or proteins) located adjacent to the peptide group of the ribosome-bound peptidyl-tRNA would be a likely candidate to be peptidyl

transferase. Attempts have been made to identify such a protein using various affinity labels.

Attempts for the identification of the protein located near the P-site, includes affinity labels such as Bromoacetyl-phe-tRNA (Pellegrini et al., 1972; Oen et al., 1973) p-nitrophenylcarbamyl-Phe-tRNA (Czernilofsky et al., 1974) p-nitrocarbamyl-Met-$tRNA_f^{Met}$ (Hauptmann et al., 1974) and bromoacetyl analogue of chloramphenicol (Sonenberg et al., 1973). All these labels bind to the 50S proteins in a poly U-dependent reaction with E. coli 70S ribosomes. Unfortunately, the results are not precise although all of them in general bind mainly to the proteins L_2 and L_{27} on the 50S particle.

According to these results it seems that L_2 and L_{27} are located near the P-site and are most probably involved in the donor site activity.

For the A-site studies puromycin analogues, N-iodoacetyl-puromycin (Pongs et al., 1973) and 5'-O-(N-bromoacetyl-p-aminophenyl-phosphoryl)3'-N-Phe-puromycin (Harris et al., 1973) have been used. Although they bind to the A site, the characterization of proteins involved with it has not been reported. Harris et al. showed however, that the binding mainly occurred with RNA.

5. Structural requirements for inhibitory and substrate activity at the Acceptor (A) site of peptidyl transferase

The observation that not only the whole molecules of tRNA but their 3'-terminal sequences are the substrates proper in the peptide forming reaction, opened the way for studying the substrate specificity and binding properties of peptidyl transferase by conventional techniques of enzymology. Whereas the investigation of simple donors of the peptide residue is only in its beginnings, the acceptor substrates have been studied more extensively.

(a) Minimum structural requirements of substrates for acceptor activity:

As stated above, the relatively simple puromycin molecule can replace the normal aminoacyl-tRNA as an acceptor substrate for peptidyl transferase. Studies have shown that the intact puromycin molecule was required for *in vitro* activity; dimethyladenine, 3'-N-(methyl-L-tyrosyl) ribose (rat liver *in vitro* protein synthesizing system (Yarmolinsky and de la Haba, 1959) puromycin aminonucleosides, adenosine, various amino acids and their esters and amides, p-nitroanilide, tyramine, and ammonium ions were all inactive as acceptors (Rychlik et al., 1969; Cerna et al., 1970a). The simplest known acceptors are A(Phe) (Rychlik et al., 1969) and A(Gly) (Cerna et al., 1970b). The acceptor activity of A(Gly) was, however, very low. At high concentrations (67%), ethanol or methanol acted as an acceptor (Jiminez et al., 1970; Vogel et al. 1970) and the reaction was stimulated specifically by CpCpA (Scolnick et al., 1970, see (f) below). It, therefore, appears

probable that water can act as an acceptor during termination of protein synthesis - a reaction believed to be carried out by peptidyl transferase (Vogel et al., 1969; Caskey et al., 1969; Capecchi and Klein, 1969).

(b) Activity of various aminoacyl analogues of puromycin and 2'(3')-O-aminoacyl adenosine.

(i) E. coli peptidyl transferase. Structure-activity studies of puromycin and its amino acid analogues (Nathans and Neidle, 1963; Waller et al., 1966; Rychlik et al., 1970; Symons et al., 1969; Harris et al., 1971) have shown that the nature of the amino acid side chain was of considerable importance for the inhibition of the synthesis of polyphenylalanine in a poly U-dependent E. coli cell-free system. Analogues containing an aromatic amino acid with a benzyl, or substituted benzyl group, in the amino acid side chain (e.g., L-phe, L-tyr and O-butyl-L-tyr derivatives) had high activity relative to puromycin while the less hydrophobic derivatives (e.g., gly, L-pro, L-Ala, L-leu, L-val and L-met) had zero or negligible activity. Essentially the same results have been obtained by using simple assay systems namely, "fragment reaction" and N-acetylPhe-tRNA-poly U systems (Eckermann et al., 1974; Cerna et al., 1970a,b). The puromycin-like activity therefore appears to increase with the hydrophobicity of the aminoacyl R group although the L-tryptophan analogue of puromycin which possesses a double aromatic ring was inactive in the poly U system (Nathans and Neidle, 1963).

There has been little study on the activity of basic and acidic analogues. 3'-N-Homocitrullyl 3-aminoadenosine was approximately

2% as active as puromycin in inhibiting cell-free protein synthesis (Guarino et al., 1963). A(Lys) had high acceptor activity in the "fragment reaction" of Monro and Marcker (1967) while A(Gly) had low, but significantly more acceptor activity than A-(Gly) Rychlik et al. 1970). Interpretation of the activity of puromycin analogues was complicated by the variation of their activity in different peptide bond forming assays (see (i) below).

(ii) Mammalian peptidyl transferase. Only fragmentary information on the substrate specificity of the acceptor site of mammalian peptidyl transferase is available. PANS-L-leu was shown by Rabinowitz and Fisher (1962) to be approximately 2.5% as effective as puromycin in inhibiting protein synthesis in Ehrlich ascites tumour cells. In addition A(Leu) and A(Phe) were equally effective inhibitors (approximately 10% as effective as puromycin) of rabbit reticulocytes in in vivo protein synthesis (Harbon and Chapeville, 1970). The extended inhibition (greater than 3 hours) of these adenosine aminoacyl esters on haemoglobin synthesis in vivo, in contrast to their low potency in vitro (due to hydrolysis of the alkali labile ester linkage) (Harbon and Chapeville, 1970) appears to be anomalous. Perhaps the compounds have secondary effects on peptidyl transferase in vivo. 3'-N-Homocitrullyl 3'-aminoadenosine was an active inhibitor of rat liver cell-free protein synthesis (Guarino et al., 1963). More recently, Eckermann et al.,(1974) have reported that the activity of the rat liver peptidyl transferase show the same general requirement for the nature of the amino acid side chain as does the E. coli enzyme. An obvious feature of

the mammalian enzyme is the generally higher activity of the hydrophobic puromycin analogues. This suggests that the rat liver ribosomes have a higher intrinsic affinity for the compounds. With the nucleotidyl analogues, both types of ribosomes show quite similar specificity patterns and favor Cp-Pan-L-Phe, the analogue most resembling the 3'-terminus of Phe-tRNA (Eckermann et al., 1974).

(c) Activity of aminoacyl isomers (D or L) of puromycin, 2'(3')O-aminoacyl adenosine and aminoacyl-tRNA. The optical configuration of the aminoacyl residue has also been shown to be important since the D-phenylalanyl analogue of puromycin (PANS-D-Phe) was far less active than the L-isomer in inhibiting the synthesis of polyphenylalanine in an E. coli poly U system (Nathans and Neidle, 1963). Of interest here is the observation that D-tyrosine (from D-tyrosyl-tRNA) was incorporated into protein at a slower rate than the L-isomer in a poly AU-dependent system from E. coli (Calendar and Berg, 1967). In contrast Rychlik et al.(1970) have demonstrated that A-D-phe was not an acceptor substrate for E. coli peptidyl transferase.

(d) Activity of various nycleoside analogues of 2'(3')-O-L-animoacyl adenosine. Rychlik et al. (1969) and Cerna et al. (1970a) have shown that the acceptor activity of the 2'(3')-O-phenylanyl ribonucleosides decreased in the sequence A. I, C while G and U were inactive. Similarly Harbon and Chapeville (1970) have shown that A(phe) had low inhibitory activity (10% of that of puromycin) on rabbit reticulocyte in vivo protein synthesis while 2'(3')-O-phenylalanyl-uridine had negligible activity.

(e) Activity of various 5'-O-substituted analogues of puromycin and 2'(3')-O-aminoacyl adenosine. Gottikh et al. (1970) have shown that the acceptor activity, as measured by the release of polyphenylalanine from E. coli ribosomes, increased in the order, A(phe), pA(phe)- methyl-pA-Phe, i.e., activity increased as the structural similarity to the 3'-terminus of aminoacyl-tRNA increased. Similarly Rychlik et al. (1967) have shown that CpA-(Gly) had high acceptor activity (but less than puromycin) while UpA-(Gly) had low activity; pA(Gly) and A(Gly) were inactive. In addition, aminoacyl-oligonucleotides (produced by T1 ribonuclease digestion of aminoacyl-tRNA) had activity equivalent to that of puromycin irrespective of the nature of the amino acid (Takanami, 1964). Similar results were reported by Symons et al. (1969) using the puromycin analogues. From the above data it is apparent that substitutions on the 5'-hydroxyl of analogues of both puromycin and 2'(3')-O-aminoacyl adenosine, which increase the structural similarity to the 3'-terminus of aminoacyl-tRNA, results in analogues with increased activity. For example, although dA(Phe) was found to be essentially inactive, (Rychlik et al., 1969), its 5'-cytidylyl derivative, CdA(Phe) was as active as CA(Phe) (Ringer and Chladek 1974b).

The studies outlined above have not distinguished between affinity of substrates for peptidyl transferase and the rate of reaction once the substrates were bound. Recently, however, Pestka et al.(1970) and Hishizawa and Pestka (1971) have demonstrated that 2'(3'-O-aminoacyl)-oligonucleotides, produced similarly

to those mentioned above, were bound strongly to E. coli ribosomes, while the binding of A(Ser) was very weak. The nature of the aminoacyl group, except in the case of the phe-oligonucleotide, had little influence, i.e., the ser, leu, val and met oligonucleotides were bound to approximately the same extent, while the phe-oligonucleotide was bound to a greater extent (approximately two-fold). The above data are consistent with the existence of a binding site, on the A site of peptidyl transferase, for the binding of the sequence CCA, which is common to all tRNA molecules. In support of this suggestion was the stimulatory activity of CCA (presumably bound to the A site), on the peptidyl transferase catalyzed reaction, of ethanol with f-Met-tRNA (Scolnick et al. 1970).

(f) Activity of 2', 3' and 5'-isomers of Puromycin and of dipeptidyl- and 2',3'-O-bis-aminoacyl derivatives of adenosine. Nathans and Neidle (1963) showed that the 2' and 5'-isomers of puromycin were inactive. A report by Pozdnyakov et al., (1972) of a significant acceptor activity elicited by 3'-O-phenylalanyl-2'-O-methyl-adenosine for peptidyl transferase indicates that the 2'-OH is not essential for acceptor activity. A-(Phe-Phe) and the 2', 3'-O-bis-aminoacyl adenosine derivatives of L-ala, L-leu and L-phe were active acceptors in the "fragment reaction" using E. coli ribosomes (Cerna et al. 1970b). The nature of the reaction products were not determined. In contrast, 3'-N-glycyl-puromycin and 3'-N-phenylalanyl-puromycin were inactive in the E. coli poly U system (Nathans and Neidle, 1963). At present the difference is not understood.

Cerna et al. (1970b) suggested that the acceptor activities of the 2', 3'-O-bis-aminoacyl adenosines indicated the possibility that 2'-3'-O-bis-substituted derivatives of tRNA may serve as intermediates during protein synthesis, as suggested earlier by Neuman et al. (1968). However, the small but significant acceptor activity of 3'-O-L-phenylalanyl-2'deoxyadenosine (Rychlik et al. 1969; Cerna et al., 1970a) indicated that the formation of the suggested intermediate was non-obligatory or, more likely, did not occur. Since CdA(phe), 2', 3'-O-bis-phenylalanyl-adenosine and 2'O-methyl-3'-O'phenylalanyl-adenosine are good acceptors, it seems reasonable to assume that the 2'-oxygen plays only an auxillary role in the binding of the acceptor substrate to the A subsite but the free hydroxyl group is not essential.

(g) Activity of puromycin analogues with alterations at the α-amino group. The α-amino group of puromycin can be modified or replaced by other nucleophiles without complete loss of acceptor activity. In this context it should be remembered that the secondary amino group of proline participates in peptide bond formation. α-Dimethylamino-puromycin was reported to be an active acceptor of nascent peptides from rat liver polysomes; in this case an unstable quaternary acylammonium linkage was presumed (Hawtrey and Biedron, 1966). This report, however, has not been confirmed. Other derivatives, α-hydroxypuromycin and 3'-N-(α-hydroxyl-L-phenylalanyl)-PANS were active acceptors (Fahnestock et al. 1970). Thus peptidyl transferase is able to form ester as well as peptide linkages. This is perhaps not surprising in view of the peptidyl transferase catalyzed attack of water on peptidyl-tRNA during

termination of protein synthesis (Vogel et al. 1969; Capechhi and Klein, 1969; Caskey et al. 1969). In this context it was of interest that methanol or ethanol acted as acceptor substrates with F-Met-tRNA as donor substrate, with the formation of N-formyl-L-methionyl-methyl ester (Vogel et al., 1970; Jimenez et al., 1970).

6. Influence of the nature of assay of Peptide bond formation on the activity of 2'(3')-O-aminoacyl adenosine and puromycin analogues. The work of Waller et al.(1966) and Nathans and Neidle (1963) have suggested that the action of puromycin in vitro protein synthesis depended, in part, on the nature of the mRNA. These workers found that puromycin, various 3'-N-aminoacyl-puromycin aminoacylnucleoside derivatives and various 2'(3') - aminoacyl adenosine derivatives were consistently more effective inhibitors of endogenous mRNA-directed in vitro protein synthesis in E. coli than poly U-directed synthesis of polyphenylalanine. Recently Rychlik et al. (1970) demonstrated that the acceptor activity of various analogues of 2'(3')-O-aminoacyl adenosine was dependent on the nature of the assay system. A(Lys) had high activity, (approximately 40% of the activity of A(Phe), with N-Ac-Phe-tRNA as the donor substrate, low activity, approximately 10% of A(Phe), with N-Ac-Leu-pentanucleotide as donor and very low, less than 1% of A(Phe), with polylysyl-tRNA as donor. Similar but less marked variations were found for the L-Ala, L-Leu and L-Ser analogues. More recently Eckermann et al. (1974) showed that Tyrosyl-im-benzyl-Histidyl, S-benzyl-cysteinyl and D-phenylalanyl derivatives of 3'-N-amino acyl analogues of puromycin had moderate activity

(17-41% of puromycin)) with N-Acetyl-phe-tRNA as the donor substrate on E. coli ribosomes but negligible activity (1-5% of puromycin) with the CpApCpCpA-(Ac-Leu) as the donor. These and other results suggest that the structural requirements for acceptor substrate activity are much less stringent in the poly-U-N-acetyl-Phe-tRNA system that in the fragment reaction.

7. Structural requirements for substrate activity at the donor (P) site of peptidyl transferase.

Only limited studies of the P site substrate specificity of E. coli peptidyl transferase have been carried out (Monro et al., 1968). As stated above, 3'-terminal fragments from f-met-tRNA can act as donor substrate (the "fragment reaction"). CAACCA(fMet), AACCA(fMet), ACCA(fMet), CCA(fMet) and CCAACCA(NAc-Met) had approximately the same activity while CA(fMet) and A(fMet) were inactive at the concentrations tested (approximately 10^{-8} M). Essentially similar results were obtained with synthetic CCA(NAc-Leu), CA(NAc-Leu) and their phenylalanine analogues (Mercer and Symons, 1972). In addition CACCA(NAc-Leu) and UACCA(NAc-Leu) had similar activity: CCA(NAc-Leu) was approximately 30% less active while CACCA(Leu) was inactive. These data indicated that a blocked amino group and the minimal sequence CCA were essential for high activity while the adjacent sequence (e.g., CCAA) were relatively unimportant and that the nature of the amino blocking group was unimportant; acetyl and formyl groups were equally effective. (See also Bretscher and Marcker, 1966).

The order of reactivity of the following fragments, AACCA(fMet) UACCA(NAc-Leu) = CACCA(NAc-Leu) CACCA(NAc-Phe), indicated that the

aminoacyl group had a marked influence on activity. This conclusion was supported by results with other N-acetyl-aminoacyl-oligonucleotides. The 2'(3')-O-(N-acetyl-L-arginyl)-oligonucleotides (produced by T1 ribonuclease digestion of N-acetyl-arg-tRNA) showed activity of the same order as CAACCA(fMet) whereas the 2'(3')-O-(N-acetyl-glycyl), and 2'(3')-O-(N-acetyl-L-aspartyl)-oligonucleotides had low and undetectable activity respectively. However, the possibility cannot be excluded that the low activities of the glycyl and aspartyl derivatives were related to their base sequences (distal to the CCA sequence) rather than their amino acid moieties. The order of activity of the various fragments was the same as the corresponding intact N-acetyl-aminoacyl-tRNA derivatives but the N-acetyl-aminoacyl-tRNA derivatives were two to four times more reactive.

Little data is available on the donor specificity of mammalian peptidyl transferase. Using human ribosomes CACCA(NAc-Leu) had activity of approximately 20% of that in the E. coli assay system, while CACCA(Leu) was inactive (Neth et al., 1970).

8. Summary of the studies of structural specificity.

In summary, the effect of four types of structural modifications of the acceptor substrates have been reported so far. They are: (1) alteration of the aminoacyl residue of the 2'(3')-O-aminoacyl nucleoside molecule, (ii) alteration at the 2' or 3'C atoms of the ribose; (iii) alteration of the heterocyclic part of the molecule and (iv) substitution on the 5'-oxygen of the ribose residue of the molecule. The dependence of acceptor activity on the first three above mentioned modifications in the acceptor substrate indicate that the acceptor substrate is fixed on peptidyl transferase at least at the first three loci and that a proper substitution at 5'-oxygen of the ribose also aids in the binding. The compound containing all three interacting components are fixed presumably ideally with respect to the catalytic center of peptidyl transferase and are therefore good acceptor substrates (e.g., A-(Phe)). If either of the weaker interacting components is absent (e.g., 2'-oxygen group of ribose in dA(Phe) or the side chain of the amino acid residue e.g.,A-(Gly)) then the substrate-moiety has a greater degree of freedom reflected by low acceptor activity, measurable only at a high concentration of acceptor substrates. Compounds lacking two interacting components (e.g., phenylalanine, phenylalanine methyl ester, tyramine, p-nitroanilide) do not show any measurable acceptor activity.

B. Purpose of thesis research

Peptidyl transferase plays a central role in protein biosynthesis. The knowledge of its specificity in terms of the structure of aminoacyl-tRNA is important in the elucidation of the detailed mechanisms of ribosomal peptide bond formation. The observation, that not the whole molecules of tRNA but their 3'-terminal sequences are the substrates proper in the peptide forming reaction, led to the use of a number of modified substrates like chemically modified AA-tRNAs and the structurally simplest donor and acceptor substrates (Rychlik et al., 1970).

During the course of this work two aspects of the specificity of ribosomal peptidyl transferase have been explored. On the one hand (1) the effect of chemical modification of the aminoacyl-adenosine end of tRNA was studied with regard to its ability to participate in the peptide bond forming process, and on the other hand (2) the effect of varying the size and secondary structure of the oligonucleotide portion of aminoacyl-oligonucleotides acting as acceptors was investigated.

1. The effect of cleavage of the 2'-3' carbon-carbon bond at the 3'-terminal adenosine of tRNA was studied by using chemically modified yeast phenylalanyl-$tRNA^{Phe}$. The modification was achieved by periodate oxidiation at pH 6 followed by $NaBH_4$ reduction at pH 8. It was reported by Ofengand and Chen (1972) that although this modified tRNA ($tRNA^{ox-red}$) was still enzymatically acylated with phenylalanine, it was unable to form a Tu-GTP-AA-tRNA ternary complex, a prerequisite for AA-tRNAs for binding to the ribosomal A site. One of the reasons suggested (Ofengand and Chen, 1972) for the lack of activity of Phe-$tRNA^{ox-red}$

was that it was the "wrong" isomer for Tu factor in terms of the exact location of the amino acid (2' or 3'). It was therefore thought this Phe-tRNA^{ox-red} might provide a convenient model to probe into the unsettled question of the stereo-chemical requirements for the peptide transfer reaction (and other partial reactions or protein synthesis) since it was known (Nathans and Neidle, 1963) that only the 3'-isomer of puromycin and not the 2'-isomer was the active acceptor substrate. It was expected that due to cleavage at the C_2', C_3' bond, the phenylalanyl moiety would be unable to undergo acyl migration. This would provide for the first time a molecular model for study of the 2' and 3' isomer specificity in peptide bond formation and in aminoacylation. Previous efforts in this field were frustrated due to the exceedingly rapid rate of acyl migration between 2' and 3' hydroxyls in aminoacyl-tRNA (Griffin et al., 1966; McLaughlin and Ingram, 1965; Wolfenden et al., 1964).

2. The structural requirements for tRNA in peptide bond formation other than a 3'-aminoacyl adenosine end is not known. Some information is available on the reactivity of f-Met-Oligonucleotides and acetyl-aminoacyl-oligonucleotides as donor substrates (Monro et al., 1968) but the acceptor activity of similar fragments was not studied. It is known that aminoacyl-oligonucleotides of undefined chain length, like aminoacylmononucleotide substrates, can also inhibit protein synthesis by forming a peptide bond with the growing polypeptide chain. Since these AA-tRNA analogues have no means of binding to the ribosomes, the resulting peptide is stripped from the ribosome and chain elongation ceases. On the other hand, AA-tRNAs not specified by the mRNA codon are unable to do so even though they possess the required aminoacyl-adenosine end

(Takanami, 1964). This inability might be attributed to the relatively larger size of the aminoacyl-tRNA molecule. Since no further characterization of the active chain lengths was reported, a careful and systematic study of the size requirements for aminoacyl-oligonucleotide stripping of polypeptide chains was undertaken. It was expected that above a certain size no reaction would occur, while below that size a very active stripping reaction would be found. A kinetic comparison in terms of relative K_m and V_{max} was thought to be a more meaningful way to show the difference in their reactivity. Such comparisons on washed ribosomes as well as on polyribosomes (a peptidyl transferase system closer to the physiological situation in the living cells) would help in better understanding the nature of ribosomal peptidyl transferase. Such attempts have been made during the course of the present work.

In summary, the present research is an effort to gain further insight into the fine details relating to the mechanism of peptide bond formation on ribosome. An attempt has been made to determine (i) which one of the two possible isomers, viz; tRNA esterified either at the 2' or 3' position, is the actual substrate in the reaction catalyzed by peptidyl transferase and (ii) what is the role of chain length and secondary structure of the acceptor substrate in peptide bond formation. The investigation reported in this thesis was directed primarily to obtain answers to these questions.

C. DISSERTATION ABSTRACT

STUDIES ON THE REACTIVITY OF RIBOSOME-BOUND PEPTIDYL TRANSFERASE FOR VARIOUS MODIFIED SUBSTRATES

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During protein synthesis peptide bond formation takes place between peptidyl-tRNA bound to the donor site and the incoming aminoacyl-tRNA at the acceptor site on the ribosome. The reaction is catalyzed by a ribosome-bound enzyme, peptidyl transferase. In the present study, two aspects of the specificity of peptidyl transferase for substrates have been explored: (1) the effect of chemical modification of the aminoacyl-adenosine end of tRNA was studied with regard to its ability to act both as a donor and as an acceptor, and (2) the effect of varying the size and secondary structure of the oligonucleotide portion of aminoacyl oligonucleotides acting as acceptor was investigated.

The effect of cleavage of the 2', 3' carbon-carbon bond at the 3'-terminal adenosine of tRNA on donor and acceptor activity was studied by using chemically modified yeast phenylalanyl-$tRNA^{phe}$. The modification was achieved by periodate oxidation at pH 6 followed by sodium borohydride reduction at pH 8. This modified tRNA ($tRNA^{ox-red}$) could still be enzymatically acylated with phenylalanine but was unable to bind with 30S ribosomal subunits in the presence of initiation factors. Although phenylalanyl-$tRNA^{ox-red}$ was bound effectively to 70S ribosomes

and specifically to the same proteins of the 50S ribosomal subunit as its equivalent control, it was totally inactive in peptide transfer reactions both as a donor and as an acceptor. It was further shown that the 3'-terminal fragments of phenylalanyl-tRNA^{ox-red} namely, CACCA^{ox-red}(Phe) and Ado^{ox-red}(Phe) were also inactive in the reaction catalyzed by peptidyl transferase. In contrast to this result, chemically synthesized Ado^{ox-red}(Phe) showed good activity. The difference in activity between synthetic and biologically prepared Ado^{ox-red}(Phe) is proposed to be due to: (a) a 2'-hydroxyl-specific enzymatic aminoacylation in tRNA^{ox-red}, (b) lack of2', 3' aminoacyl migration in the ring-opened structure, and (c) a specificity of peptidyl transferase and of initiation factors for 3'-aminoacyl esters. These results indicate that the 2'-hydroxyl group on tRNA is the primary site of esterification for aminoacyl-tRNA synthetases.

The effect of size on acceptor site activity was investigated by a comparative kinetic study of AAUCCUUCCCCCACCACCA-Tyr, ACCA-Tyr and A-Tyr. The relative K_m and V_{max} for these substrates in the peptidyl transferase reaction were determined using both a model system (N-acetyl-Phe-tRNA bound to salt-washed E. coli ribosomes) as well as a more physiological system (washed E. coli polysomes carrying nascent peptidyl-tRNA). With salt-washed ribosomes, the K_m depended on their chain lengths being 125, 8 and 20 μM, for the 1-, 4- and 19mer respectively, while using washed polysomes, the K_m varies in a different way being 4.5, 7.5 and ca 400 μM. Thus with salt-washed ribosomes, the monomer has an anomously high K_m while with polysomes, the 19mer is the one which shows a greatly reduced affinity. The differential reactivity observed for different size oligomers suggest that peptidyl transferase may indeed

be shielded by the structure of the polyribosomes from attack by the bulky aminoacyl-tRNA in the cytoplasm. In order for peptide bond formation to occur, the enzyme site must be made available to the aminoacyl acceptor end of the incoming codon-specified aminoacyl-tRNA possibly by induced conformational changes in the ribosomes. In this way, non-mRNA specified aminoacyl-tRNAs would be excluded from acting as peptide chain acceptors. The effect of secondary structure was also examined by complexing AAUCCUUCCCCCACCACCA-Tyr in which nine of the twelve residues normally base-paired are cytidine, with $(I_p)_5I$. It was observed that the formation of a structure analogous to the amino acid acceptor stem of the tRNA cloverleaf did not affect the activity of 19mer-Tyr on polysomes.

CHAPTER II

A. Materials

Unfractionated E. coli tRNA and bakers' yeast tRNA were purchased from Schwarz BioResearch; brewers' yeast $tRNA^{phe}$ (1200 pmole/A_{260} unit) from Boehringer-Mannheim, E. coli Q13 frozen cells (mid-log phase) from General Biochemicals and fresh yeast cells from Red Star Corp. (Belleville, N.J.). Poly U and $(Ip)_5I$ were obtained from Miles Laboratories. Tetracycline-HCl was a gift from Chas. Pfizer and puromycin-HCl was purchased from Nutritional Biochemicals. Trityladenosine, t-BOC-Phenylalanine and N-hydroxysuccinimide ester of phenoxyacetic acid were from Schwarz-Mann. Phenylalanyl-phenylalanine was purchased from Cyclo Chemical Company and N-acetyl-phenylalanine from Mann Research Laboratories.

Pyruvate kinase, PEP, DTT, and T_1 RNase were obtained from Calbiochem (Los Angeles). DNase I (electrophoretically pure), pancreatic RNase and eggwhite lysozyme were purchased from Worthington Biochemical Corp. Polynucleotide kinase was a gift from Dr. A. Nussbaum of the Chemical Research Department of Hoffmann-La Roche. Sephadex G-25, G-50 and DEAE-Sephadex A-25, were purchased from Pharmacia Fine Chemicals, DEAE-cellulose and CM-cellulose were obtained from Bio-Rad Laboratories, and BD-cellulose was obtained from Schwarz BioResearch. Cellulose nitrate filters (type HAWP and BDWP) were from Millipore Corporation. Glass fiber filter paper discs (Whatman GF/C), and Whatman No. 52 and 3 MM chromatography paper were from H. Reeve Angel & Company.

Silica gel and Cellulose TLC sheets were purchased from Eastman Kodak Company and Merck Avicell Cellulose and Silica gel preparative (2 mm) plates were obtained from Brinkman Instruments, Inc., Westbury, N.Y.) All TLC materials contained a fluorescent indicator.

N-acetyl-Phenylalanyl-Phenylalanine was synthesized by acetylating phenylalanyl-phenylalanine with acetic anhydride as described by Greenstein and Winitz (1961). 0.4 ml of acetic anhydride at the rate of 0.1 ml at each 15 min. interval were added to a solution of 100 micromoles (212 mg) of phenylalanyl-phenylalanine in 0.4 ml of glacial acetic acid at 0^{o} C. The total incubation time was one hour. The solvent was evaporated under reduced pressure at room temperature and the residue dissolved in acetic acid. The compound was tested by TLC (R_f, N-acetyl-Phe-Phe 0.93; N-acetyl-Phe 0.85; Phe-Phe 0.81 on cellulose sheet in butanol:acetic acid:water, 4:1:1) and by electrophoresis as in Figure 15 against Phe-Phe and N-acetyl-Phe (Phe-phe, + 3 cm; N-Ac-Phe, + 24.2 cm product, + 16.6 cm).

L-[^{14}C]Tyrosine (395 mCi per mmole), generally labelled L-[^{3}H]-Phenylalanine (2 Ci per mmole), [methoxy-^{3}H]Puromycin dihydrochloride (740 mCi per mmole), [^{3}H]Acetic anhydride (400 mCi per mmole) and [^{32}P]-ATP (19 Ci per mmole) were obtained from New England Nuclear. tBOC-L-[^{14}C]Phenylalanine (75 mCi per mmole) and L-[^{3}H]Phenylalanine (G) (50.5 Ci per mmole) were from Schwarz-Mann, L-[^{14}C]phenylalanine (475 mCi per mmole)was from Amersham-Searle. L-[^{3}H]Amino Acid mixture (average specific activity 330 mCi/mmole) was obtained from Schwarz Bio-Research.

B. Preparation of Peptidyl Transferase Substrates.

1. Preparation of mixed [^{3}H]aminoacyl-oligonucleotides.

a. Preparation of mixed [^{3}H]aminoacyl-tRNA

Aminoacylation of E. coli crude tRNA was carried out according to the method of Henes et al. (1969). The reaction mixture contained, in a final volume of 24 ml, 100 mM 3,3-dimethylglutaric acid buffer pH 6.9, 10 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 193 A_{260} units of unfractionated tRNA, an excess of E. coli aminocyl-tRNA synthetases (Muench et al., 1966) and 0.6 mM each of 20 [^{3}H] amino acids (average specific activity 0.18 mCi/mmole. The conditions for optimal incorporation of amino acid into tRNA in terms of incubation time and the amount of enzyme was determined in separate experiments. After incubation at 37° C for 30 minutes, 0.1 volume of 20% potassium acetate, pH 5, was added, the reaction mixture was chilled to 0° C and shaken at 4° C for 5 minutes with an equal volume of phenol saturated with 10 mM sodium acetate, pH 5. The aqueous layer was removed and the phenol layer was washed with an equal volume of 10 mM sodium acetate buffer, pH 5. tRNA was precipitated from the combined aqueous layers with 2 volumes of cold 95% ethanol. After 6 hours at -30° C, the precipitated tRNA was recovered by centrifugation, dissolved in the 10 mM acetate buffer, and reprecipitated. The final precipitate was dissolved in a minimum volume of 10 mM sodium acetate, pH 5, and desalted on a Sephadex G-25 column. The [^{3}H]aminoacyl-tRNA peak was pooled, and precipitated by the addition of 0.1 volume of 20% potassium acetate, pH 5, and 2.5 volumes of cold 95% ethanol. The final precipitate was recovered by centrifugation and dissolved in 2 ml of 0.01 M sodium acetate buffer pH 5. It was dialyzed against water for 2 hours before storage at -30° C. All of the above

operations were carried out at 4° C except as noted.

b. Preparation of T_1-RNase products of [^{3}H]aminoacyl-tRNA

The method was a modification of the procedure described by Takanami (1964). The reaction mixture for the digestion of aminoacyl-tRNA contained in a total volume of 1 ml, 50 mM sodium acetate buffer, pH 5.5, 2 mM EDTA, 100 units of T_1-RNase, and 66.3 A_{260} units of [^{3}H]aminoacyl-tRNA. The amount of enzyme was determined by conducting pilot experiments using graded amounts of enzyme to convert the [^{3}H]aminoacyl-tRNA added into an acid soluble fraction during 10 minutes incubation at 37° C. Following the incubation at 37° C for 10 minutes, the reaction mixture was chilled and shaken twice with cold phenol saturated with buffer (10 mM sodium acetate, pH 5.5) and the aqueous layer separated. Phenol was removed from the aqueous layer (1 ml) by two ether extractions and the aqueous layer was used after removing ether by gently blowing nitrogen through it.

c. Preparation of Pancreatic RNase digestion products of [^{3}H]aminoacyl-tRNA

Conditions used for pancreatic RNase digestion of [^{3}H]-aminoacyl-tRNA were similar to those described by Preiss et al. (1959). The reaction mixture (1 ml) contained 67.5 A_{260} units of [^{3}H]aminoacyl-tRNA, 50 mM sodium acetate buffer, pH 5.5, 2 mM sodium EDTA, and 0.3 mg of pancreatic RNase. The amount of enzyme was just sufficient to convert the added aminoacyl-tRNA into an acid soluble form in 10 minutes at 37° C as determined by separate experiments. The reaction mixture was incubated for 10 minutes at 37° C, then treated with phenol and recovered as described in the case of T_1-RNase digestion.

2. Purification of E. coli $tRNA^{Tyr}$

a. Purification of uncharged tRNA

E. coli $tRNA^{Tyr\ I\ \&\ II}$ was purified by two cycles of BD-cellulose chromatography as described by Gillam et al. (1971) for yeast $tRNA^{Tyr}$. In the first cycle, a BD-cellulose (50-100 mesh) column (2.5 x 50 cm) previously equilibrated at 4^{o} C with 10 mM NaOAc, pH 5.5, 10 mM $MgSO_4$, 10 mM mercaptoethanol, and 0.3 M NaCl was loaded with 56,000 A_{260} units of crude uncharged E. coli tRNA dissolved in 300 ml of the equilibrating buffer. Fractionation was achieved with a linear salt gradient obtained with 2 liters of the equilibrating buffer in the mixer and 2 liters of 1.9 M NaCl in 10 mM NaOAc, pH 5.5, 10 mM $MgSO_4$, 10 mM mercaptoethanol in the reservoir. The column was run at 4^{o} C and 7.5 ml fractions were collected at a flow rate of 30 ml per hour. At the end of the gradient, the column was flushed with 1.5 M NaCl, 10% ethanol, in 10 mM NaOAc, pH 5.5, 10 mM $MgSO_4$, 10 mM mercaptoethanol. Fractions were assayed for tyrosine acceptance in a final volume of 0.25 ml according to the procedure described for large scale charging below, and the concentration of NaCl was determined by conductivity. Two peaks of tyrosine acceptance activity were detected (Fig. 1A). The first peak eluted in the salt gradient as a broad peak and had 55% of the total tyrosine acceptance while the second peak was eluted by 10% ethanol and had 29% of the total tyrosine acceptance.

The overall recovery of tRNA in terms of tyrosine acceptance activity was 84%. All fractions with tyrosine acceptance activity (peaks I and II) were pooled, and precipitated with 2.5 volumes of cold ethanol, dissolved in water, and dialyzed against water for six hours. The extent of purification in the salt and the ethanol pooled fractions were 8-fold

Figure 1

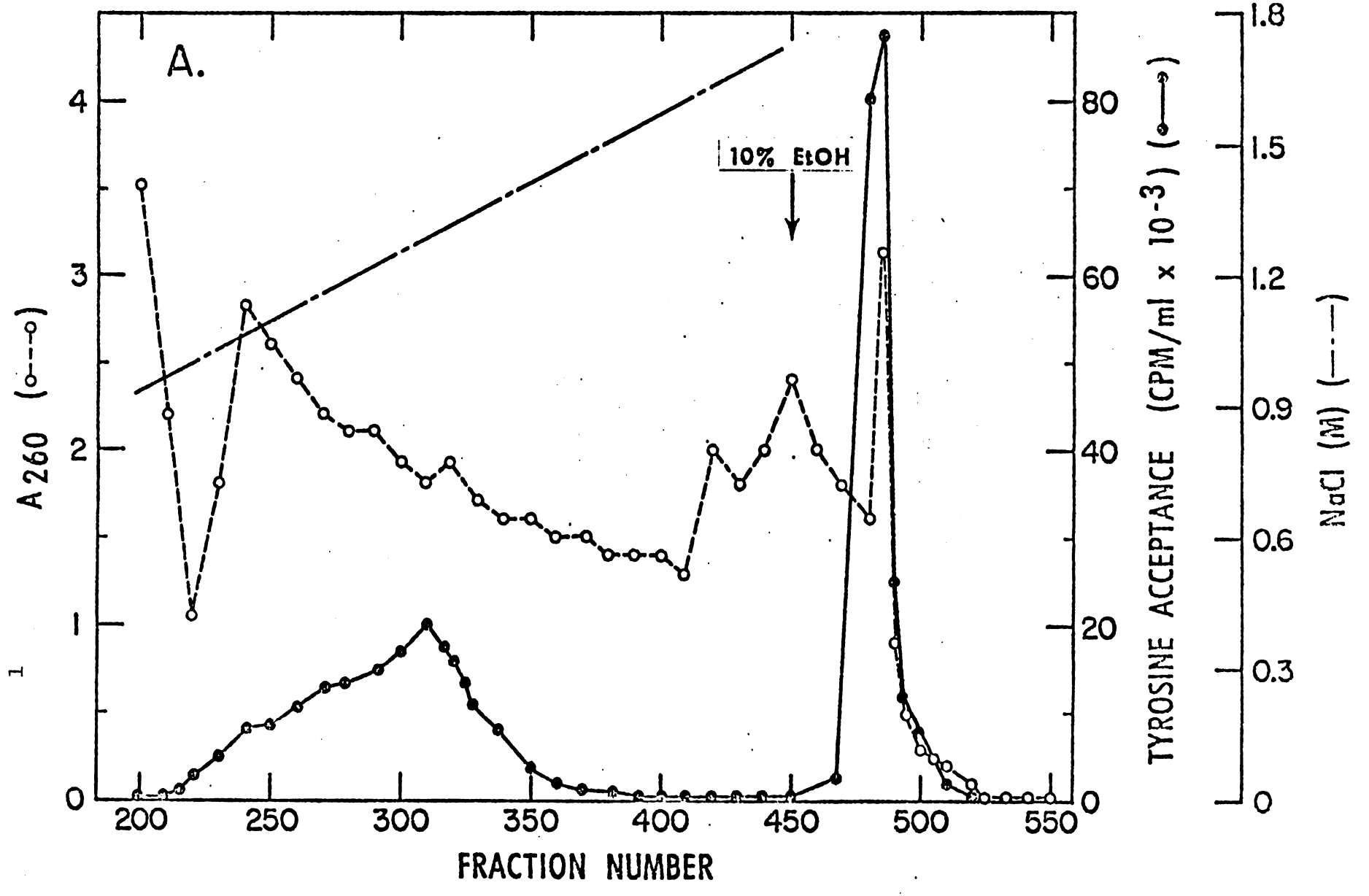
Large scale purification of $tRNA^{Tyr}$

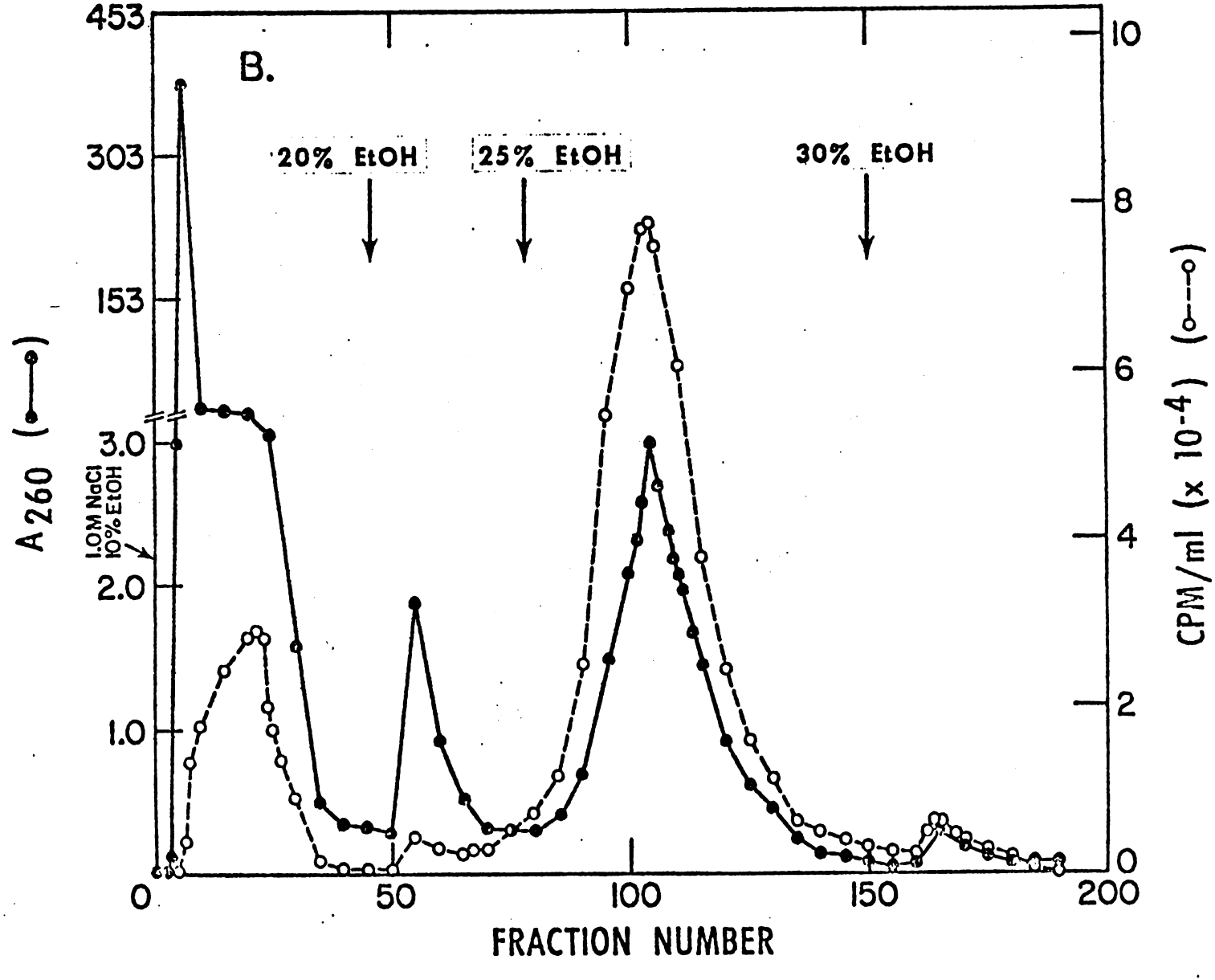
A. Chromatography of uncharged E. coli tRNA on BD-Cellulose

Details of the procedure are described in Chapter II.B.2.a. Only the latter part of the elution profile is shown. Most of the A_{260} is eluted between 0.35 and 0.6 M NaCl. Eighty-four percent of the total input $tRNA^{Tyr}$ was recovered as $tRNA^{Tyr}$. ○-○ , A_{260}; ●-● , [^{14}C]Tyrosine acceptance; ------, NaCl concentration. Fractions #220-360 and 465-510 were pooled for processing in B.

B. Chromatography of N-phenoxyacetyl[^{14}C]Tyr-$tRNA^{Tyr}$ on BD-Cellulose

Details of the procedure are described under purification of $tRNA^{Tyr}$ in Chapter II.B.2.a. The column was run at a flow rate of 90 ml per hour and 15 ml fractions were collected. The arrows indicate the changes of ethanol concentration. Fractions #90-130 were pooled. The recovery of the total input radioactivity was 96%. ○—○ , A_{260}; ●—●, [^{14}C]Tyrosine cpm.


A.
10% EtOH
A 260 (o---o)
TYROSINE ACCEPTANCE (CPM/ml x 10-3) (o——o)
NaCl (M) (——)
FRACTION NUMBER
0
1
2
3
4
0
20
40
60
80
0
0.3
0.6
0.9
1.2
1.5
1.8
200
250
300
350
400
450
500
550


B.
20% EtOH
25% EtOH
30% EtOH
1.0M NaCl
10% EtOH
A260 (—●—)
CPM/ml (x 10-4) (o---o)
FRACTION NUMBER
453
303
153
3.0
2.0
1.0
0
50
100
150
200
10
8
6
4
2
0

and 4.5-fold, respectively. Further purification of this enriched tRNA preparation was achieved by a second cycle of BD-cellulose column chromatography of the charged tRNA (tyrosyl-tRNA) after derivatization with the phenoxyacetic ester of N-hydroxy succinimide.

b. Preparation of Tyrosyl-tRNA

Large scale charging of $tRNA^{Tyr}$ was carried out in a reaction mixture containing 100 mM 3,3-dimethylglutaric acid buffer, pH 6.9, 10 mM MgOAc, 10 mM KCl, 2 mM ATP, 3926 A_{260} units of enriched tRNA, 0.04 mM [^{14}C]tyrosine (10 μc/μmole) and 540 mg of unfractionated E. coli aminoacyl-tRNA synthetase (Henes et al., 1969) in a final volume of 200 ml. This amount of enzyme and tRNA gave complete charging of the tRNA in 45 minutes at 37° C. Assessment of maximal charging was done by withdrawing aliquots at various times, and stopping the reaction with 3 ml of cold 5% TCA. After 10 minutes at 0° C, the precipitate was collected on Millipore filters, washed with cold TCA (3 x 5 ml), dissolved in Bray's solution (Bray, 1960), and counted at 92% efficiency. The large scale incubation was stopped by the addition of 0.1 volume of 20% KOAc, pH 5.2 and chilling to 0° C. After extraction with an equal volume of phenol saturated with 2% KOAc, pH 5.2, the aqueous phases were combined and 2.5 volume of 95% cold ethanol were added. After 6 hours at -20° C, the tyrosyl-tRNA was centrifuged, dissolved in 32 ml of 0.1 M triethanolamine hydrochloride buffer, pH 4.0. Final recovery of the [^{14}C]tyrosyl-tRNA was 92%.

c. Preparation of N-phenoxyacetyl-tyrosyl-tRNA

The [^{14}C]tyrosyl-tRNA prepared above in 0.1 M triethanolamine hydrochloride (pH 4.1) was cooled to 0° C, and to the stirred

solution was added 225 mg of the phenoxyacetic ester of N-hydroxy-succinimide in 1.5 ml of dry tetrahydrofuran. Much of the added material precipitated. The pH of the mixture was quickly adjusted to 8.0 by addition of 1 N sodium hydroxide and stirring was continued at 0^{o} C. After five minutes, an additional 150 mg in 1 ml of tetrahydrafuran was added and the pH maintained at 8.0. At the end of 10 minutes, glacial acetic acid was added to pH 4.5 and the RNA was recovered by precipitation with two volumes of cold 95% ethanol. The precipitate was washed twice with cold ethanol and carefully drained. The extent of derivatization was estimated by hydrolyzing the derivatized material with 0.3 M NaOH for 15 minutes at 37^{o} C followed by paper chromatography. 5 µl (20,000 cpm) of the hydrolysate was spotted on 3 MM paper and chromatographed in butanol:acetic acid:water (78:5:17). After drying, 1-cm strips were counted under toluene scintillation fluid. Phenoxyacetyl-Tyr-tRNA and Tyr-tRNA stayed at the origin. The R_f values for phenoxyacetyl-tyrosine and free tyrosine were 0.92 and 0.22 respectively.

d. Chromatography of N-phenoxyacetyl-tyrosyl-tRNA on BD-cellulose.

The derivatized material was chromatographed on a BD-cellulose (100-200 mesh) column (1.5 x 30 cm) previously equilibrated in 1.0 M NaCl, 10 mM NaOAc, pH 5.5, 10 mM $MgSO_4$, 10 mM mercaptoethanol and 10% ethanol at 4^{o} (Krauskopf et al., 1972). A solution of 3329 A_{260} units (1.2×10^{6} pmoles of [^{14}C]tyr-tRNA) in 150 ml of equilibrating buffer was loaded onto the column and washed with the same buffer to elute underivatized Tyr-tRNA (22% of the total). The column was washed with buffer containing 20% ethanol until the eluate had negligible A_{260}. The ethanol concentration was then increased to 25% to elute the phenoxyacetyl

derivative as a symmetrical peak with a pooled specific activity of 1490 pmoles per A_{260} (85-95% pure) (Fig. 1B).

After ethanol precipitation, hydrolysis of the phenoxyacetyltyrosine residue from the tRNA was accomplished by incubation in 1.8 M Tris-HCl, pH 8.5. At 37° C the half-life was 1.4 minutes and 6 minutes incubation was routinely used for stripping. For the determination of the half-life, phenoxyacetyl-[^{14}C]tyr-tRNA was incubated as above. Aliquots were removed at every minute up to 7 minutes, precipitated with 5% TCA at 0° C, collected on Millipore filters and counted to determine the phenoxyacetyl[^{14}C]tyr-tRNA remaining. The zero time value was obtained by extrapolation of the first order decay curve. This treatment left 4% of the tRNA acylated and inactivated 20% of the remaining chains for subsequent tyrosine acceptance. At the end of the stripping reaction, the pH was adjusted to 4.5 by adding glacial acetic acid and discharged tRNA was recovered by precipitation with 2.5 volume of cold 95% ethanol at 20° C for 6 hours. The tRNA was dissolved in 0.2 M KOAc, pH 5, and reprecipitated with 2.5 volumes of ethanol. The final precipitate was dissolved in 2 mM NaOAc, pH 5.5, and 5 mM $MgCl_2$ and dialyzed against the same buffer for 10 hours. The tRNA was stored in liquid nitrogen.

3. Preparation of AAUCCUUCCCCCACCACCA[^{14}C]Tyr (19mer[^{14}C]Tyr) from *E. coli* Tyr-$tRNA^{Tyr}$

a. Digestion with T_1 RNase and Isolation of 19mer-Tyr by DEAE-Sephadex A-25 Column Chromatography

The reaction mixture for the complete digestion of Tyr-tRNA, in 15 ml, contained 93 A_{260} units (1×10^5 pmoles) of pure *E. coli* [^{14}C]Tyr-tRNA (1975 pmole/A_{260};200 cpm/pmole), 10 mM Tris-acetate, pH 7.0,

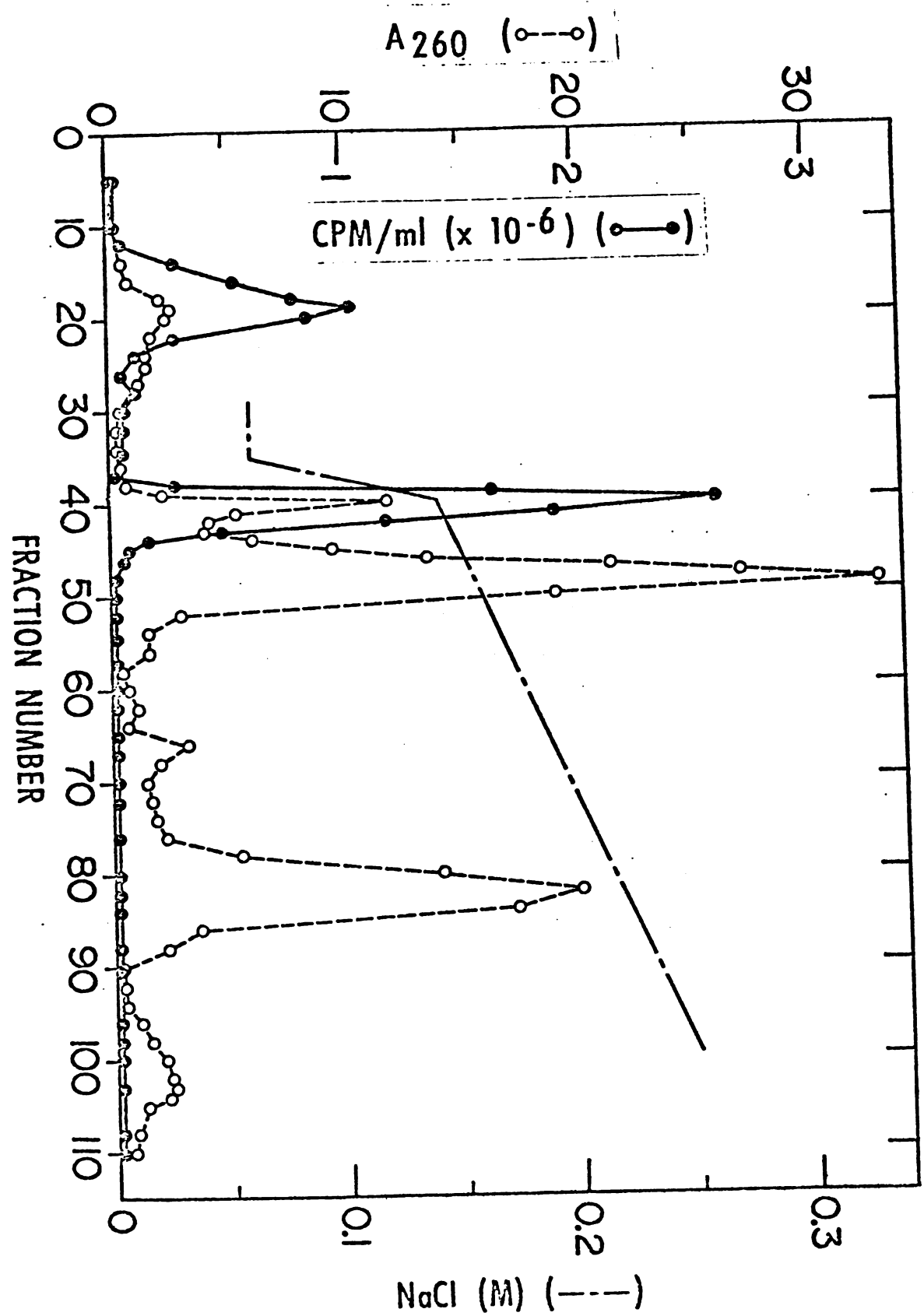
5 mM EDTA and 564 units of T_1 RNase. Incubation was for 20 minutes at 37° C. Pilot experiments showed these conditions resulted in complete digestion as judged by column chromatography as described below. 19mer-Tyr was isolated according to the method described by Beltchev and Grunberg-Manago (1970). At the end of the incubation, 6.3 gm of solid urea was added to the reaction mixture to a final concentration of 7 M and it was quickly adjusted to pH 3.0 with 6 N HCl. The entire mixture was placed onto a DEAE-Sephadex A-25 column, washed with 5 ml of 7 M urea-HCl, pH 3.0, and eluted with a NaCl gradient in 7 M urea-HCl, pH 3.0 as shown in Figure 2.

The initial small peak of radioactivity was acid soluble and corresponded to free tyrosine liberated under the conditions of digestion and chromatography. It was estimated by independent experiments that under these conditions of digestion, 20 per cent of the bound tyrosine was deacylated. The main peak of radioactivity was acid insoluble and eluted at the beginning of the gradient which was expected of the largest oligomer (Beltchev and Grunberg-Manago, 1970). The total acid precipitable counts in this peak represented 76 per cent of the digested input radioactivity. The pooled fractions indicated in Figure 2 represented 38 per cent of the input counts and the total recovery from the column was 96 per cent. 0.1 volume of 20 per cent potassium acetate, pH 5.0, was added to fractions 38-41 and the fragment isolated from urea solution by precipitation with three volumes of cold ethanol overnight at -20° C. The precipitate was collected by centrifugation and dissolved in a minimum volume of 0.01 M ammonium formate, pH 5.0. It was dialyzed against the same buffer for two hours at 0° C and stored at -170° C.

Figure 2

Chromatography of a T_1-RNase digest of *E. coli* $Tyr\text{-}tRNA^{Tyr}$ on DEAE-Sephadex A-25 in 7 M urea-HCl, pH 3.0

A complete T_1-RNase digest (Chapter II.B.3.a) of $Tyr\text{-}tRNA^{Tyr}$ (1 x 10^5 pmoles) in 7 M urea-HCl, pH 3.0, was applied to a column (0.5 x 105 cm) of DEAE Sephadex A-25 which was eluted at 23° C with a NaCl gradient in 7 M urea-HCl, pH 3.0 as indicated. Fractions of 1.5 ml were collected at a flow rate of 15 ml per hour. Tubes #38-41 were pooled. ○—○ A_{260}; ●—●, [^{14}C]Tyr; ---------, NaCl concentration.


A260 (o--o)
CPM/ml (x 10-6) (o—o)
FRACTION NUMBER
NaCl (M) (---)
0
10
20
30
40
50
60
70
80
90
100
110
0
0.1
0.2
0.3
1
2
3

b. Characterization of 19mer-Tyr fragment

The purified 3'-terminal 19mer-Tyr was identified by its elution position from DEAE-Sephadex-urea at pH 3.0 where its preponderance of A and C residues causes it to elute early, and by its insolubility in both acid and 75% ethanol. Additional characterization of the fragment was achieved by end group analysis at the 5' end using [^{32}P]phosphate incorporation by polynucleotide kinase (Hänggi et al., 1970) followed by snake venom phosphodiesterase treatment (Stoltzfus and Banerjee, 1972). According to the known sequence (Goodman et al., 1970) of E. coli tRNA^{Tyr}, the 19mer from the 3' end contains 5 A residues which are located at positions 1,4,7,18 and 19 from the 3' end. Identification of A at the 5' end of the fragment by the above method would indicate that the fragment was either 1,4,7,18 or 19 nucleotides long. The possibility that any of the first three chain lengths were correct could be excluded from the knowledge that they would be soluble in acid (5% TCA) since less than 1 per cent of the similar pentanucleotide CACCA-Phe is precipitable by cold 5% TCA (Pestka et al., 1970). Since the isolated fragment containing bound tyrosine was completely precipitable by 5% TCA, it was reasonable to assume that the fragment was larger than a heptanucleotide. Identification of A as the 5' terminal nucleotide was achieved as follows.

The [^{32}P]phosphate incorporation mixture contained in 0.1 ml 100 mM Tris-acetate pH 7.4, 10 mM magnesium acetate, 25 mM β-mercaptoethanol, 0.2 mM γ-^{32}P-labeled ATP (1197 cpm/pmole), 0.063 A_{260} units (359 pmoles of precipitable [^{14}C]tyrosine) of polynucleotide and 2 units of polynucleotide kinase. Incubation was carried out at 37° C for 40 minutes at which time 88% of the polynucleotide fragment was labelled with radioactive phosphate. The amount of fragment added or phosphate incorporated

was estimated by the measurement of TCA precipitable [^{14}C] or [^{32}P] cpm. The incubation was terminated with 0.1 volume of 20% potassium acetate, pH 5.2, and the fragment was isolated by precipitation with 4 volumes of cold ethanol for 6 hours at -20° C. It was dissolved in 0.1 ml of 2 mM Tris-HCl, pH 7.0, and desalted on a Sephadex G-15 column (0.9 x 15 cm) to remove excess [^{32}P[ATP. [^{32}P]labelled polynucleotide fragment eluted at 0.38 column volumes and was clearly separated from free ATP (eluted at 0.51 of column volumes).

Hydrolysis of the labelled polynucleotide into its constitutent mononucleotides was achieved by treatment with snake venom phosphodiesterase (Stoltzfus and Banerjee, 1972). The digestion mixture in 0.1 ml contained 100 mM Tris-HCl, pH 8.4, 5 mM $MgCl_2$, 2 µg snake venom phosphodiesterase and 33 pmoles of 5'-[^{32}P]labelled-fragment (40,000 cpm). It was incubated at 37° C for one hour. The reaction mixture was passed through a pinch of Dowex 50 (H^+ form) in a Pasteur pipette to remove salts from the nucleotides and was washed with 0.1 ml of water (Stoltzfus and Banerjee, 1972). The nucleotide sample was lyophilized and applied to a 50 cm long strip of Whatman No. 3 paper. 5'-UMP, 5'-GMP, 5'-CMB, 5'-AMP and ATP were added as markers. Electrophoresis was carried out in pyridine-acetate buffer, pH 3.5 (Pyridine:Acetic Acid:H_2O; 1:10:89) for 2 hours at 3000 volts. At the end of the electrophoresis, the paper was dried in a warm stream of air and then placed in a tank saturated with ammonia vapors to neutralize the pyridine in order to reduce its absorption of ultraviolet light. The paper was dried in air again and was then scanned under ultraviolet light and the absorbing spots of the markers were marked. For the analysis of the experimental sample, strips of the paper 1 cm wide were cut and the radioactivity counted under 10 ml of

toluene scintillation fluid (Figure 3). Eighty-three per cent of the radioactivity migrated to the position corresponding to that of 5'-AMP marker and the remaining 17% to that of 5'-UMP. It is likely that the fast moving radioactive spot (17%) was due to inorganic phosphate derived from residual phosphatase activity on the γ-^{32}P-ATP and/or the labelled fragment. In any case, at least 83% of the fragment contains A at its 5' end. This confirms that the isolated Tyr-fragment is an oligonucleotide of approximately the correct length. The ratio of 3' to 5' ends based on tyrosine attached per residue of [^{32}P] incorporated was 1.14.

Based on the amount of labelled tyrosine attached per A_{260} of the fragment it was estimated that the fragment was 82% acylated assuming zero hypochromicity. The exact extent of hypochromicity is not known.

4. Preparation and Characterization of ACCA-[^{14}C]Tyr from yeast Tyr-tRNA.

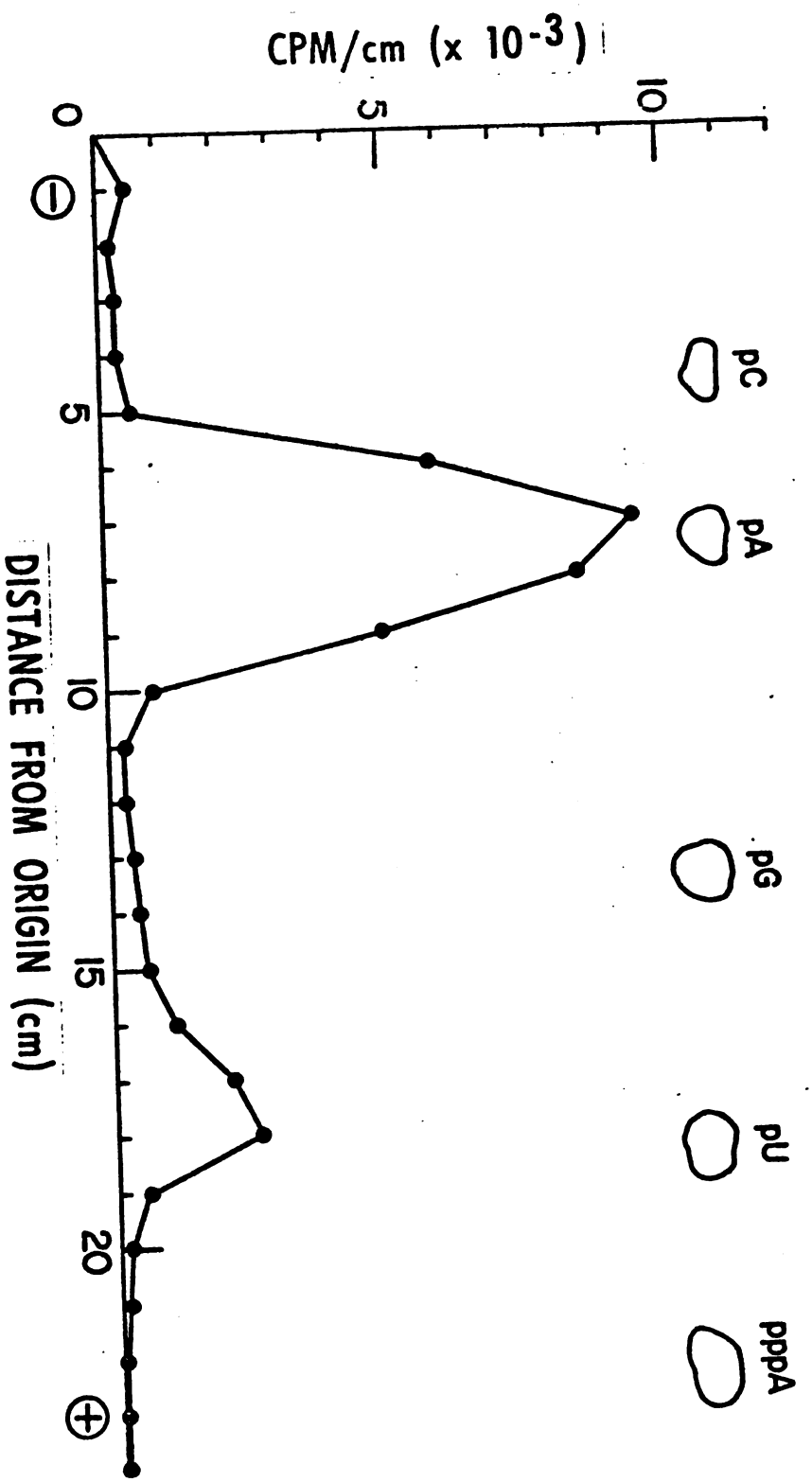
Yeast Tyr[^{14}C]tRNA was prepared according to the method described in Chapter II.B.2.b. for E. coli Tyr-tRNA except that crude yeast aminoacyl-tRNA synthetases were used. The reaction mixture contained 100 mM 3,3-dimethyl-glutaric acid buffer, pH 6.9, 10 mM $Mg(OAc)_2$, 10 mM KCl, 2 mM ATP, 1400 A_{260} units of unfractionated yeast tRNA (45 pmole/A_{260} unit), 0.05 mM[^{14}C]Tyrosine (100 mc/mmole) and crude yeast aminoacyl-tRNA synthetases in a final volume of 80 ml. The amounts of enzyme and tRNA were adjusted to give complete charging. The mixture was incubated at 30^{o} C for 50 minutes and the tRNA was recovered from the reaction mixture as described in Chapter II.B.2.b.

Figure 3

Identification of the 5'-nucleotide derived from digestion of 5'-[^{32}P]labeled polynucleotide fragment

Details of the procedure are described in Chapter III.B.3.b. 40,000 cpm of 5'-[^{32}P]labeled fragment were electrophoresed on Whatman 3 MM paper in pyridine-acetate buffer, pH 3.5, for 2 hours at 3,000 volts.

●—● , [^{32}P]-containing 5'-nucleotide.


CPM/cm (x 10-3)
0
5
10
⊖
5
10
15
20
⊕
DISTANCE FROM ORIGIN (cm)
pC
pA
pG
pU
pppA

ACCA-[^{14}C]Tyr was prepared by a modification of the method described by Pestka et al. (1970). The reaction mixture for complete digestion of Tyr-tRNA^{Tyr} contained 1100 A_{260} units (5×10^4 pmoles) of yeast [^{14}C]Tyr-tRNA^{Tyr} (200 cpm/pmole); 10 mM potassium acetate, pH 5.2; 5 mM sodium EDTA and 2500 units of T_1 RNase in a total volume of 6.6 ml. Incubation was for 30 minutes at 37° C, and resulted in the conversion of more than 90% of the [^{14}C]tyrosine to acid soluble form. At the end of incubation the entire mixture was chromatographed on a DEAE-Sephadex A-25 column (Figure 4). [^{14}C]Tyr-oligonucleotide emerged in a single homogeneous peak at about 0.61 M ammonium formate. The fact that the radioactivity peak did not coincide with the peak of A_{260} corresponding to the ACCA-Tyr was probably due to the fact that unfractionated tRNA was used (3% tRNA^{Tyr}), so that many unacylated polynucleotides of similar chain length were present in the preparation.

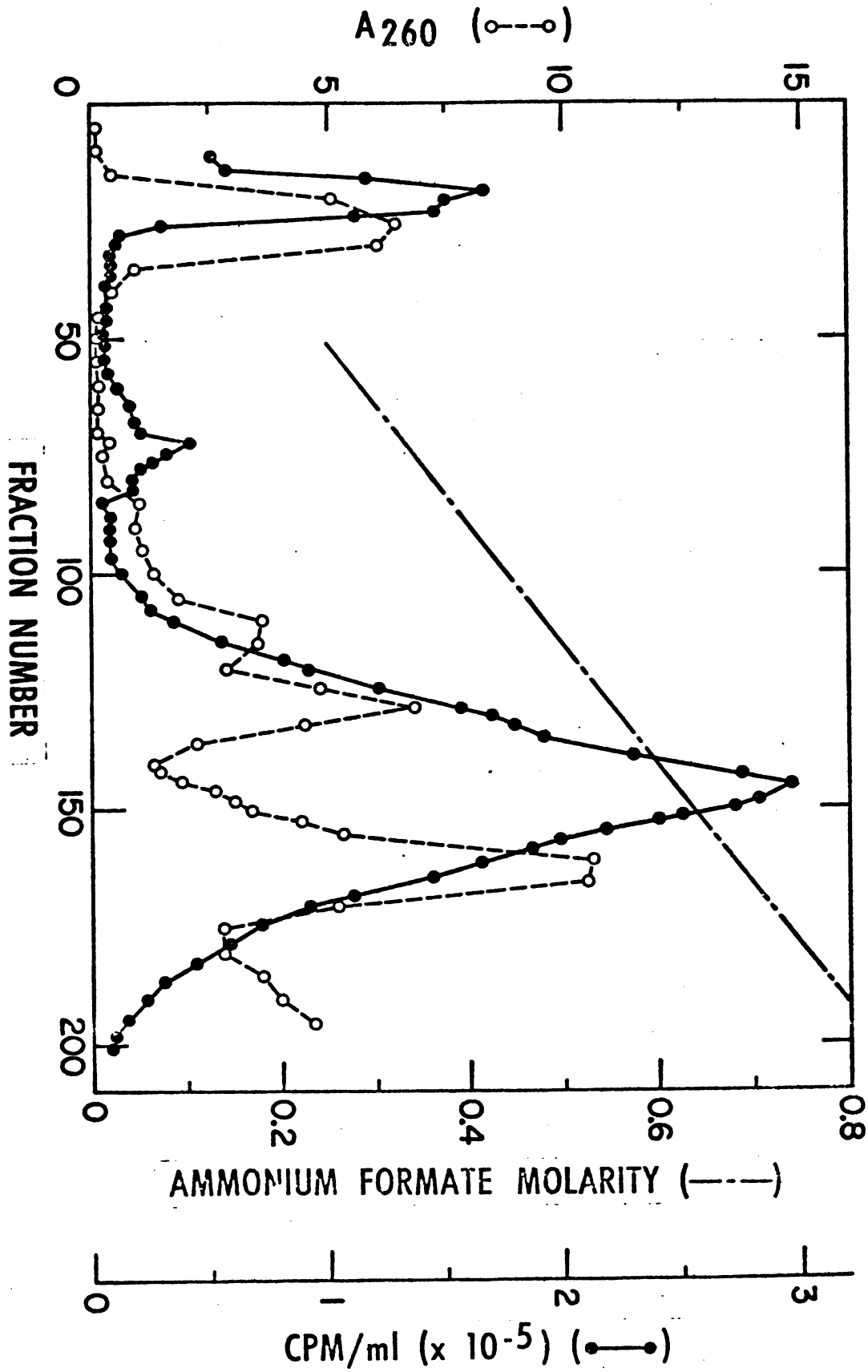
From the specificity of T_1 ribonuclease and a knowledge of the sequence of yeast tRNA^{Tyr} (Goodman et al., 1966), it is presumed that the [^{14}C]Tyr-oligonucleotide has the sequence ACCA(Tyr). The fractions were pooled, freeze dried, dissolved in water, again freeze dried to remove residual ammonium formate, and finally dissolved in a minimum volume of 2 mM ammonium formate, pH 5.0. Further purification, as well as characterization of the tetramer fragment was achieved by electrophoresis on Whatman No. 3 MM paper in pyridine-acetate buffer, pH 3.5 (0.25% pyridine and 2.5% acetic acid) for 3 hours with a voltage gradient of 50 volts per cm in the Savant high voltage electrophorator (Pestka et al., 1970). The purified ACCA-Tyr was detected by its tyrosine radioactivity and the coincident UV absorbing band. Under these conditions, the major radioactive spot moved 5.5 cm to the cathode and was separated from free tyrosine (mobility 7.5 cm) and T_1 RNase which stayed

Figure 4

Chromatography of a T_1-RNase digest of yeast Tyr-$tRNA^{Tyr}$ on DEAE-Sephadex A-25

A complete T_1-RNase digest of yeast Tyr-$tRNA^{Tyr}$ (5×10^4 pmoles), Chapter II.B.4. was applied to a DEAE-Sephadex A-25 column (1.5 x 24 cm) which was previously equilibrated with 0.01 M ammonium formate pH 5.0. The column was washed with 80 ml of the above buffer to remove free tyrosine, and Tyr-oligonucleotides were eluted with a linear gradient from 0.2 to 0.8 M ammonium formate pH 5.0 (total volume 400 ml). The column was run at 4° C. Fractions of 2 ml were collected at a flow rate of 30 ml per hour. Tubes #135-151 were pooled. The recovery of acid precipitable radioactivity as [^{14}C]Tyr-oligonucleotide was 84%.

○—○ , A_{260}; ●—● [^{14}C]Tyrosine; ------, ammonium formate concentration.


A260 (o---o)
0
5
10
15
FRACTION NUMBER
0
50
100
150
200
0
0.2
0.4
0.6
0.8
AMMONIUM FORMATE MOLARITY (——)
0
1
2
3
CPM/ml (x 10-5) (•—•)

at the origin. A standard sample of CACCA(Phe) (kindly supplied by Dr. S. Pestka), under the same conditions, migrated 4.9 cm. The ACCA-(Tyr) spot was eluted with 0.01 M ammonium-formate, pH 5.0, lyophilized and finally dissolved in 0.01 M ammonium-formate, pH 5.0 and stored at -170° C. The final specific activity of the ACCA-Tyr was 24,060 pmoles per A_{260} (70% acylation assuming zero hypochromicity). Before each experiment, the sample was checked for hydrolysis by silica gel TLC in the upper phase of butanol:ethanol:water (4:1:5) at 4° C (R_f, ACCA-(Tyr) - 0.0, R_f, tyrosine - 0.32).

5. Preparation and Characterization of Adenosyl-[^{14}C]Tyrosine.

Adenosyl-[^{14}C]tyrosine was prepared by digesting 325 A_{260} units of yeast [^{14}C]Tyrosyl-tRNA (8.2×10^4 pmoles) prepared as described above with 1.5 mg of pancreatic ribonuclease. Enriched tRNA (254 pmole/A_{260} unit) was obtained after BD-Cellulose chromatography of unfractionated yeast tRNA according to the method described in Chapter II.B.2.a. This enriched fraction was eluted with 0.85 NaCl with a total recovery of 93% of the input $tRNA^{Tyr}$ activity. The reaction for enzymatic digestion was carried out at 30° C for 15 minutes in 0.02 M triethylammonium-formate buffer, pH 4.6, in a total volume of 6.3 ml. In preliminary small scale experiments the release of adenosyl-tyrosine was complete under these conditions of incubation as assessed by the retention of radioactivity on CM-cellulose under the chromatographic conditions described below. The separation method was a modification of the chromatographic step described by Waller et al. (1966).

Immediately after incubation, the mixture was chilled, adjusted to pH 2.9 by the addition of 0.1 M formic acid and applied to a CM-

cellulose column (1 x 12 cm) previously equilibrated with 0.01 M formic acid, pH 2.9 at 4° C. Fractions of 2 ml were collected at a flow rate of 90 ml per hour. Free [^{14}C]tyrosine, nucleotides, and some adenosine were eluted by washing the column with 0.01 M formic acid. The concentration of formic acid was then raised to 0.2 M which eluted most of the A-[^{14}C]Tyr (92% of input radioactivity). The fractions (total 60 ml) were pooled and the recovered material (2.12 absorbance unit at 260 nm) was immediately freeze-dried and stored at -170° C. The specific activity of the final preparation was 31.7 nmoles per A_{260} (47% of theory). The above chromatographic procedure did not remove all free adenosine.

Ado(Tyr) was characterized by its UV absorption spectrum (Waller et al., 1966) TLC on silica gel in n-butanol:ethanol:water (4:1:5) at 22° C or 4° C [R_f at 22° C; Ado(Tyr) = 0.7; Tyr = 0.35; R_f at 4° C; Ado(Tyr) = 0.56; Tyr = 0.32]. The estimation of Ado-[^{14}C]Tyr was routinely done by TLC at 4° C which caused negligible hydrolysis during chromatography.

Ribonuclease remained tightly bound to the column under the above conditions. It was not released by 0.5 M formic acid and could be recovered by using 0.9 M formic acid as eluant. The absence of RNase in the 0.2 M formic acid effluent containing Ado(Tyr) was further ascertained by the following control experiment. A column identical to that used for the preparative run was loaded with 3 mg of pancreatic RNase alone and subjected to the chromatographic procedure described. The 0.2 M formic acid effluent corresponding in volume to that containing Ado(Tyr), was pooled and lyophilized. It was examined for its inhibitory effect on the charging of pure yeast $tRNA^{Phe}$. The amount of phenylalanine charged was identical to that of a control sample confirming the absence of ribonuclease in the fraction which would contain Ado(Tyr), although

when the 0.9 M formic acid effluent was tested likewise, it inhibited completely.

6. Preparation of [^{14}C]Phe-tRNA^{ox-red} and N-acetyl[^{14}C]Phe-tRNA^{ox-red}.

a. Preparation of yeast phenylalanine tRNA^{ox-red}

Oxidation and reduction of tRNA^{Phe} was carried out as described by Ofengand and Chen (1972). Periodate oxidation of yeast tRNA^{Phe} (1200 pmole/A_{260}) and of Phe-tRNA^{Phe} (1112 pmole/A_{260}) prepared as described for Phe-tRNA^{ox-red} below (section b), was performed in a solution containing 0.1 M NaOAc, pH 5.5; 10 mM $MgCl_2$; 7-30 A_{260} units per ml of tRNA, and 4 mM $NaIO_4$ for 30 minutes at room temperature in the dark. At the end of the incubation, a 1.5-fold excess of glucose or rhamnose to periodate was added and the mixture further incubated for 5 minutes in the dark to destroy the excess periodate. The oxidized tRNA was isolated by ethanol precipitation (2.5 vol), washed once by solution in 0.2 M KOAc, pH 5.5, reprecipitated with 2.5 ml ethanol and finally dissolved in water. 100% oxidation was achieved as shown by the complete loss of charging activity.

Reduction of the $NaIO_4$-oxidized tRNA to the diol was performed using sodium borohydride. A 5 mg/ml solution of $NaBH_4$ in 0.2 M potassium phosphate buffer, pH 7.8 (initial pH after addition of $NaBH_4$, 9.5) was adjusted to pH 8.1 at room temperature with acetic acid. One volume of this solution was added to one volume of oxidized tRNA (27-30 A_{260} unit per ml) and incubated for 2 hours at room temperature in the dark. After two hours, an additional 0.5 volume of $NaBH_4$ solution was added and the reduction reaction continued for an additional 2 hours. The modified

tRNA was isolated by precipitation with 2.5 volume of cold ethanol and washed by two reprecipitations from 0.2 M potassium acetate, pH 5.5, with 2.5 volume of cold ethanol. It was then dialyzed against four liters of 2 mM potassium acetate buffer, pH 5.5, 10 mM $MgCl_2$ with two changes for 16 hours at 4° C to remove residual salts. Reduction of $NaIO_4$-treated Phe-$tRNA^{Phe}$ was performed similarly after a prior incubation in 0.4 M Tris-chloride, pH 9.0, 0.5 M KCl at 37° C for 30 minutes to remove phenylalanine. The tRNA was isolated by precipitation with 2.5 volume of cold ethanol. The stripping reaction should not be applied to the oxidized unacylated $tRNA^{Phe}$ since β-elimination at this pH removes the terminal A residue (Brown et al., 1955).

It was observed that if the oxidized $tRNA^{Phe}$ was treated with $NaBH_4$ solution at around pH 7.5, the degree of regeneration of acceptance activity was increased. In this way 94% (average of three experiments) of the original acceptance activity could be restored after reduction. Therefore, in later experiments, the procedure was modified as follows: 10 µl of glacial acetic acid was added to 5 ml of a 5 mg/ml solution of $NaBH_4$ in 0.2 M potassium phosphate buffer, pH 7.1, at 0° C (pH of the resulting mixture was 7.5 at 22° C as checked separately) and the reduction solution immediately mixed with an equal volume of the oxidized $tRNA^{Phe}$ solution. After 2 hours incubation at room temperature in the dark, the same volume of freshly prepared $NaBH_4$ solution was added again and the incubation continued for an additional two hours. The oxidized-reduced-$tRNA^{Phe}$ was then isolated as described above.

b. Acylation of $tRNA^{ox-red}$ with phenylalanine

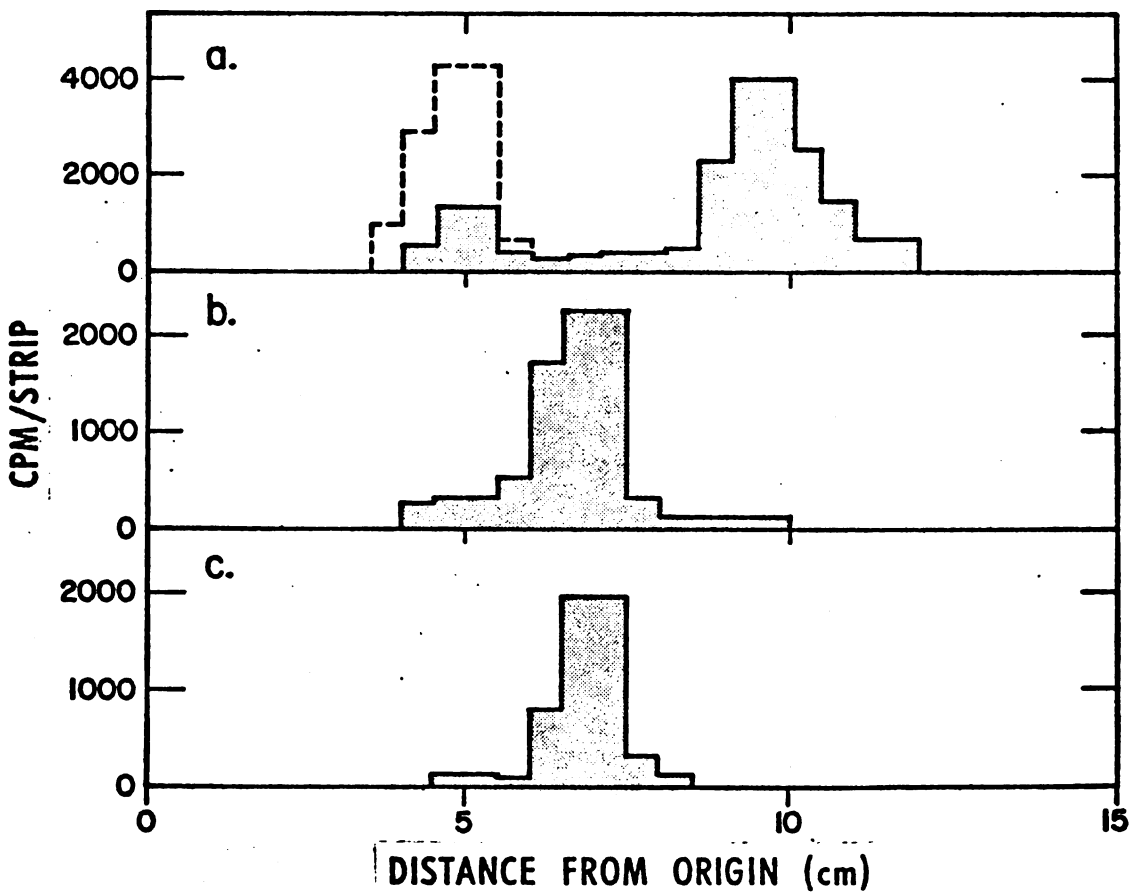
The aminoacylation of tRNA and $tRNA^{ox-red}$ with phenylalanine using a mixed yeast AA-tRNA synthetase was performed according to

the procedure described by Ofengand and Chen (1972). The charging mixture contained the following components: 100 mM 3,3-dimethylglutaric acid buffer, pH 6.9, 10 mM KCl, 10 mM $Mg(OAc)_2$, 2 mM ATP, 20 μM [^{14}C]phe (50 μC/μmol), 2-3 A_{260} units of tRNA or $tRNA^{ox-red}$ per ml, and crude yeast charging enzyme. The amount of enzyme and the time of incubation were adjusted to give maximal charging of $tRNA^{ox-red}$. After incubation at 37° C the charged $tRNA^{ox-red}$ was recovered by phenol extraction, ethanol precipitation and G-25 Sephadex column chromatography as described in chapter II.B.2.b. The final average charging specific activity of $tRNA^{ox-red}$ was 80-90% of the control. Because of the possibility of replacement of the 3' terminal Ado^{ox-red} by Ado due to the combined action of nucleases and CCA pyrophosphorylase possibly contaminating the charging enzyme preparation, the nature of the 3' ends was directly examined by RNase hydrolysis and chromatographic characterization of the resulting Ado(Phe) derivatives at 4° C (Figure 5). According to the results, 5% of the Ado^{ox-red}(Phe) radioactivity is in the region corresponding to Ado(Phe). However, if the TLC was run at 22° C which gave much better separation of these compounds (R_f:Ado^{ox-red}(Phe) = 0.85; Ado(Phe) - 0.40), only one per cent of the radioactivity of Ado^{ox-red}(Phe) was found at the position of Ado(Phe). The preparation of synthetic Ado^{ox-red}(Phe) shown in panel C of the figure will be discussed below (Chapter II.B.10.). The ratio of adenosine to phenylalanine for these compounds was determined from the ratio of UV absorption to radioactivity. Ado:Phe was 1·8 for Ado(Phe) and 1·3 for Ado^{ox-red}(Phe).

Figure 5

Chromatographic Analysis of Ado(Phe) and Ado^{ox-red}(Phe) derived from their respective Phe-tRNA with synthetic Ado^{ox-red}(Phe)

18,000 cpm of Ado[^{14}C]Phe (panel a), 10,650 cpm of Ado^{ox-red}[^{3}H]Phe (panel b), both derived by treatment of their respective Phe-tRNAs with 25 µg pancreatic RNase for 10 minutes at 37° C (see Chapter II.B.6.b.) and 4,000 cpm of chemically synthesized Ado^{ox-red}[^{14}C]Phe (panel c) in 1 mM ammonium formate buffer, pH 5.0, were applied to a silica gel thin layer chromatography sheet and chromatographed at 4°C in the upper phase of the solvent n-butanol-ethanol-water (4:1:5 v/v). The chromatogram was dried, cut into 1-cm strips and counted under 10 ml of toluene scintillation fluid. R_f values are Ado(Phe) = 0.63; Ado^{ox-red}(Phe) = 0.46; Phe = 0.33 (dashed line, panel a).


a.
b.
c.
4000
2000
0
2000
1000
0
2000
1000
0
0
5
10
15
CPM/STRIP
DISTANCE FROM ORIGIN (cm)

c. Acetylation of Phenylalanyl-tRNA, Phe-tRNA^{cont} and Phe-tRNA^{ox-red} with acetic anhydride.

The method was as described by Haenni and Chapeville (1966). To a solution of Phe-tRNA or Phe-tRNA^{ox-red} (2900 to 4600 pmoles/ml; 830-966 pmoles/A_{260} unit) in 5 mM KOAc, pH 5.2; 10 mM $Mg(OAc)_2$, an equal volume of saturated potassium acetate buffer, pH 5.0 was added. The mixture was maintained at 0° C, and a total of 0.1 volume of acetic anhydride was added to the mixture in four equal volumes over the period of 30 minutes. One-half volume of water was then added followed by 2.5 volumes of cold ethanol. The mixture was kept at -20° C for 2 hours, then centrifuged. The tRNA was dissolved in 5 mM KOAc, pH 5.2, and reprecipitated with 2.5 volume of cold ethanol. The final precipitate was dissolved in 5 mM KOAc, pH 5.2, 10 mM $Mg(OAc)_2$ and stored at -170° C.

The extent of acetylation was determined by hydrolysis of the acetylated Phe-tRNA with 0.3 N NaOH at 37° C for 30 minutes followed by descending chromatography for 18 hours, in n-butanol:acetic acid:water (78:5:17) on Whatman 3 MM paper. Radioactivity on the paper was located by counting 1-cm strips in toluene scintillation fluid. The extent of acetylation was calculated as the ratio of counts moving faster than free phenylalanine to the total counts applied. Distances (in cm): Phe, 6.0; untreated reaction mixture, 0.0; alkali hydrolyzed reaction mixture, 13.0. Using this method, 95-98% of the Phe-tRNA was found to be acetylated.

For acetylation with [^{3}H] acetic anhydride. the procedure was slightly modified. 100 µl of [^{14}C]Phe-tRNA (290 pmoles Phe-tRNA^{cont}; 319 pmoles of Phe-tRNA^{ox-red}) was added to 50 µl of saturated potassium

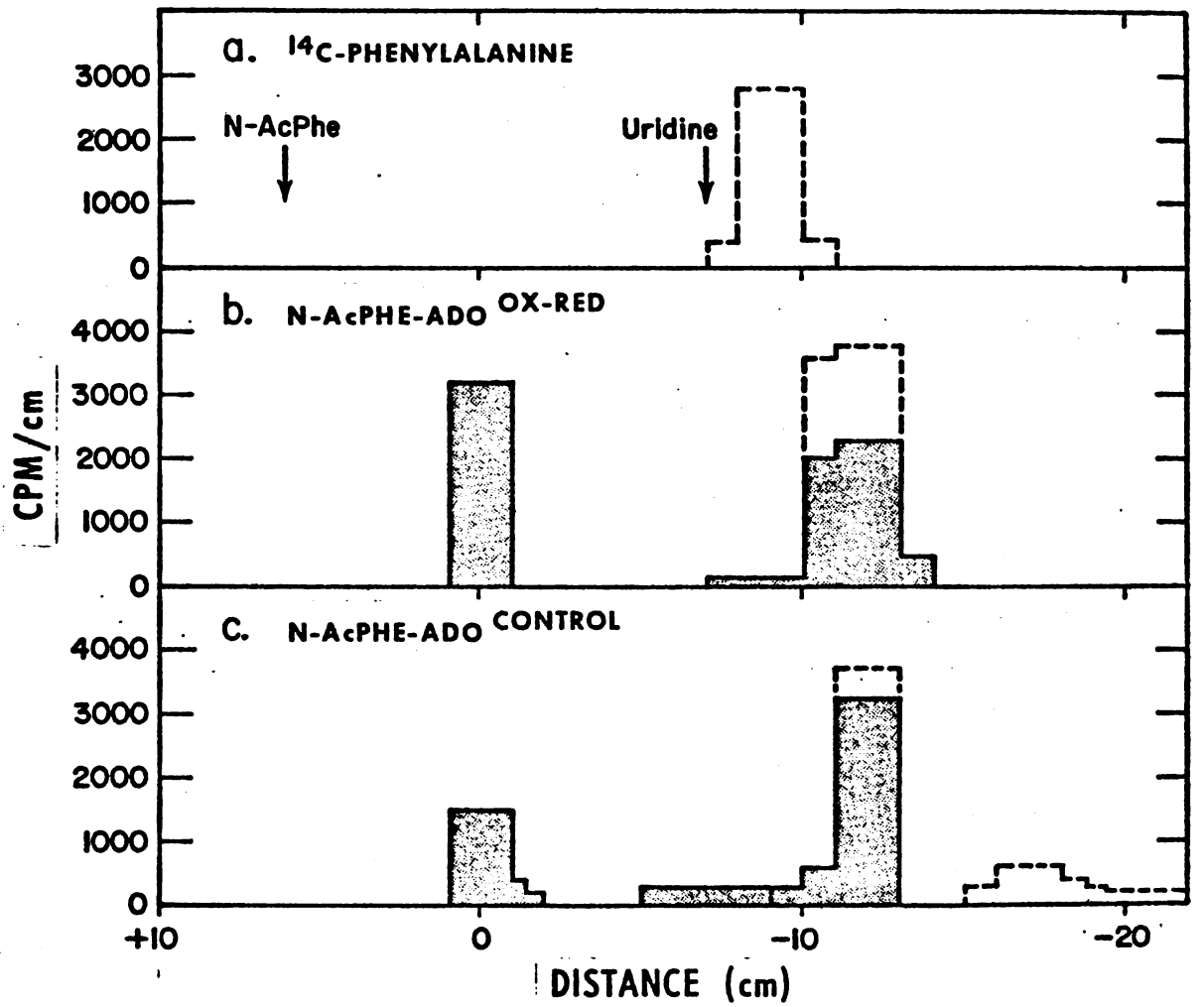
acetate buffer, pH 5.2. 100 µl of [^{3}H] acetic anhydride (400 mc/mmole) was added at 0° in 25 µl portions over a 30 minute period; 750 µl of cold ethanol was then added and the mixture kept at -20° C for 6 hours. The precipitate was dissolved in 200 µl of 2% potassium acetate, pH 5.2, and again precipitated with three volumes of cold ethanol for 6 hours at -20° C. Finally the acetylated Phe-tRNA was dissolved in 70 µl of 1 mM sodium acetate buffer, pH 5.5 and the location of [^{3}H]acetyl groups determined after RNase digestion by paper electrophoresis (Figure 6). This procedure was considered necessary since chemical acetylation of aminoacyl-tRNA^{ox-red} had not been described previously, and it was possible that the Phe-tRNA^{ox-red} was acetylated at other loci (e.g., pentose hydroxyls, ring amino group, etc.) instead of at the primary amino group of the phenylalanine residue. As shown in Figure 6, both oxidized-reduced and control [^{14}C]Phe-tRNAs yielded similar acetylated products (obtained after RNase digestion of the acetylated Phe-tRNAs) which according to their mobility at pH 3.5 are clearly N-[^{3}H]acetyl-[^{14}C]Phe-adenosine compounds. Also, the ratio of [^{3}H]acetyl/[^{14}C]Phe in both these compounds (0.80 for oxidized-reduced and 0.86 for control) show that they are monosubstituted acetylated products. Monosubstitution either at Ado-NH_2 or at the ribose hydroxyl would produce a compound having an extra positive charge at pH 3.5. These data therefore, rule out the possibilities of acetylation at Ado-NH_2 and ribose hydroxyl and clearly suggest that it is the amino group of the phenylalanyl moiety which is acetylated.

Figure 6

Analysis of acetylated Phe-tRNA^{ox-red} by paper electrophoresis of the pancreatic RNase digestion products.

Acetylation was performed with [^{3}H]acetic anhydride as described in Chapter II.6.c. 20 µl of the acetylated products were treated with 4 µg of pancreatic RNase and incubated for 10 minutes at 37°. The mixture was applied to Whatman 3 MM paper and electrophoresed at 3000 volts (60 volts/cm) for 3 hours in 1% acetic acid, 0.1% pyridine, pH 3.5. The paper was air-dried, cut into 1 x 1.5 cm sections and counted under toluene scintillation fluid. N-acetylphenylalanine and uridine marker positions are indicated by the arrows.

[^{3}H], closed bar; [^{14}C], open bar


a. 14C-PHENYLALANINE
N-AcPhe
Uridine
b. N-AcPHE-ADO OX-RED
c. N-AcPHE-ADO CONTROL
CPM/cm
3000
2000
1000
0
4000
+10
0
-10
-20
DISTANCE (cm)

7. Preparation and characterization of CACCA-(Phe) and CACCA(N-acetyl-Phe) from Phe-tRNA and Phe-tRNA^{ox-red}.

CACCA^{ox-red}-[^{14}C]Phe and CACCA-[^{14}C]Phe were prepared by a modification of the procedure of Herbert and Smith (1967) as described by Pestka et al. (1970). All operations for isolation of the pentanucleotide fragments bearing the phenylalanine residue were carried out at 4° C. Reaction mixtures for the digestion of the respective [^{14}C]Phe-tRNAs contained the following components: 4.0 A_{260} units of [^{14}C]Phe-tRNA^{cont} (3.6 x 10^3 pmoles) or 4.5 A_{260} units of [^{14}C]Phe-tRNA^{ox-red} (4 x 10^3 pmoles), 0.01 M potassium acetate, pH 5.0, 0.002 M sodium EDTA, and 7.5 units of T_1-RNase in a final volume of 0.2 ml. Incubations were carried out at 37° C. After 30 minutes the reaction mixture was placed on a column of DEAE-Sephadex (0.5 x 2 cm) which had been washed with 5 ml of 5 M ammonium formate, pH 5.0 and then equilibrated with 0.01 M ammonium formate, pH 5.0. Ammonium formate (0.01 M) was used to elute any free [^{14}C]phenylalanine. The CACCA-[^{14}C]Phe was lyophilized and dissolved in a minimum volume of 1 mM ammonium formate, pH 5.0 and stored at -170° C until used. As reported by Pestka et al. (1970) less than 1% of the isolated [^{14}C]Phe-oligonucleotide was precipitable with cold 5% trichloroacetic acid.

Preparation of the N-acetyl derivatives was achieved by T_1-RNase digestion of the respective N-acetyl-Phe-tRNAs as described above except that 3.8 A_{260} units of N-acetyl-[^{14}C]Phe-tRNA^{cont} (2.6 x 10^3 pmoles) or 2.77 A_{260} units of N-acetyl-[^{14}C]Phe-tRNA^{ox-red} (1.7 x 10^3 pmoles) were used. Chromatography of the T_1-RNase digests were performed as indicated above. However the CACCA-(N-acetyl-Phe) derivatives required

0.45 M ammomium formate, pH 5.0 for elution (Pestka, et al., 1970). The fractions were pooled, lyophilized, dissolved in 1 mM ammonium formate, pH 5.0 and stored at -170° C prior to use. Only 0.8% of the isolated fragment was precipitable with cold 5% trichloroacetic acid.

The fragments (all four) thus obtained were characterized by high voltage electrophoresis on Whatman No. 52 paper in 0.5% pyridine-5% acetic acid buffer, pH 3.5, for 3 1/2 hours with a voltage gradient of 40 volts/cm in the Gilson High Voltage Electrophoretor. Standards of CACCA[^{3}H]Phe and CACCA-(Ac[^{3}H]Phe) (kindly supplied by Dr. S. Pestka) were also run. The oligonucleotide fragments were detected by means of the radioactive amino acid label. After electrophoresis, the paper was dried, cut into 1-cm pieces and counted under 10 ml of toluene scintillation fluid. The mobility of all the fragments were identical to those of their respective standards. CACCA([^{14}C]Phe) moved -5.1 cm and CACCA-(Ac-[^{14}C]Phe) moved + 1 cm.

8. Preparation and Characterization of Ado-(Phe) and Ado^{ox-red}(Phe)

Ado-[^{14}C]Phe was prepared by pancreatic RNase digestion of yeast [^{14}C]Phe-tRNA (397 pmole/A_{260}), 100 cpm/pmole), 680 A_{260} units (2.7×10^5 pmoles) of [^{14}C]Phe-tRNA prepared as described in Chapter II. B.6.b in 4.5 ml of triethylammonium formate buffer, pH 4.6, was treated with 0.6 mg of pancreatic RNase for 15 minutes at 30° C in a total volume of 4.6 ml. The reaction was stopped by addition of 3 ml of 0.01 M formic acid. Ado(Phe) was isolated from the digest by CM-cellulose column chromatography as described in Chapter II.B.5. with a final recovery of 83%.

Ado^{ox-red}(Phe) was prepared from purified Brewer's yeast [^{3}H]Phe-tRNA^{ox-red} (1052 pmole/A_{260}, 200 cpm/pmole) as above. 19 A_{260} units of [^{3}H]Phe-tRNA^{ox-red} (2×10^4 pmoles) in 0.9 ml of triethylammonium formate buffer, pH 4.6, was treated with 20 µg of pancreatic RNase in a final volume of 1 ml. The mixture was incubated for 15 minutes at 30° C, 1 ml of 0.01 M formic acid was added, and Ado^{ox-red}(Phe) was isolated by CM-cellulose column chromatography as described above. The final recovery was 88%.

Ado(Phe) and Ado^{ox-red}(Phe) were characterized by thin layer chromatography at 4° C on silica gel in the upper phase of n-butanol: ethanol:water (4:1:5) as described by Ofengand and Chen (1972), (Figure 5). The concentrations of these compounds when used in peptide transfer assays were freshly determined by TLC analysis as above with correction of the concentration for the amount of free phenylalanine observed. The same chromatographic assay was used to determine the amount of Ado(Phe) in the Ado^{ox-red}(Phe) preparation. As shown in

9. Preparation and characterization of N-bromoacetyl-Phe-$tRNA^{ox-red}$ and N-bromoacetyl-Phe-$tRNA^{cont}$

N-bromoacetyl[^{3}H]Phe-$tRNA^{ox-red}$ was prepared by the treatment of [^{3}H]Phe-$tRNA^{ox-red}$ with N-bromoacetylsuccinimide according to the procedure of Pellegrini et al. (1972). [^{3}H]Phe-$tRNA^{ox-red}$ (1020 pmoles per A_{260} unit) was obtained by $NaIO_4$ oxidation, $NaBH_4$ reduction and acylation of brewer's yeast $tRNA^{Phe}$ (1200 pmoles per A_{260} unit) as described in section 6.a. and b. of this chapter, except that 0.54 μM [^{3}H]Phe (40 Ci/mmole) was used, 5.0 A_{260} units of $tRNA^{ox-red}$ was charged in a 1.2 ml reaction volume, and BD-cellulose chromography was substituted for the Sephadex gel filtration step. The ethanol precipitate was dissolved in 0.5 ml of 10 mM NaOAc, pH 5.5, 0.1 M NaCl, 10 mM $MgCl_2$ and loaded onto a BD-cellulose column (0.5 x 8 cm). Free phenylalanine and ATP were removed by washing with the same buffer containing 0.2 M NaCl. The column was then washed with 1.0 M NaCl to remove uncharged tRNA. Finally, the [^{3}H]Phe-$tRNA^{ox-red}$ was eluted from the column by buffer containing 1.0 M NaCl and 15% ethanol, and recovered by precipitation with 2.5 volumes of cold ethanol at -30° C overnight. The precipitation step was repeated and the final precipitate was dissolved in 10 mM NaOAc, pH 5.2. The specific activity of the final preparation was 500 pmoles per A_{260} unit.

The control [^{3}H]Phe-tRNA was prepared similarly. 10 A_{260} units of $tRNA^{Phe}$ was first charged with phenylalanine and then subjected to the $NaIO_4$ oxidation conditions. Phenylalanine was removed from the tRNA as described in section 6.a. of this chapter and the stripped

tRNA was then exposed to the reduction conditions used above for making $tRNA^{ox-red}$. Finally, it was charged with [^{3}H]phenylalanine (40Ci/mmole) using the same components and conditions as for the $tRNA^{ox-red}$ described above. The same procedure was followed for the separation of the charged tRNA and yielded a Phe-tRNA with a final specific activity of 594 pmoles per A_{260} unit. The apparent reason for the low extent of charging was the lower concentration of the [^{3}H]phenylalanine used for aminoacylation, since it was not possible to have the optimal concentration (0.03 M) keeping the desired high specific activity required for the particular experiment. The BD-cellulose chromatography step was only partially successful in separating uncharged $tRNA^{Phe}$ from Phe-$tRNA^{Phe}$.

Bromoacetylation of the Phe-tRNAs was carried out by Ms. Marie Pellegrini in the laboratory of Dr. Charles R. Cantor, Department of Chemistry and Biological Sciences, Columbia University. The N-hydroxysuccinimide ester of α-bromoacetic acid was prepared using a procedure analogous to that of de Groot et al. (1966). The product of this reaction was dissolved in dimethylsulfoxide and added in a 2:1 weight ratio to a solution of [^{3}H]Phe-$tRNA^{ox-red}$ or [^{3}H]Phe-$tRNA^{cont}$ (105 pmole/ml) in 50 mM potassium phosphate buffer, pH 6.8. The mixture was incubated at 37° C for 3 hours and the respective bromoacetyl[^{3}H]Phe-tRNAs were recovered after addition of 0.1 volume of 20% potassium acetate, pH 5.2, by ethanol precipitation with 2.5 volume of cold ethanol at -30° C for 6 hours. The precipitation step was repeated twice, and the final precipitate was dissolved in 10 mM sodium acetate buffer, pH 5.5 and dialyzed against water for 4 hours at 4° C. The final product of the reaction was approximately 70%

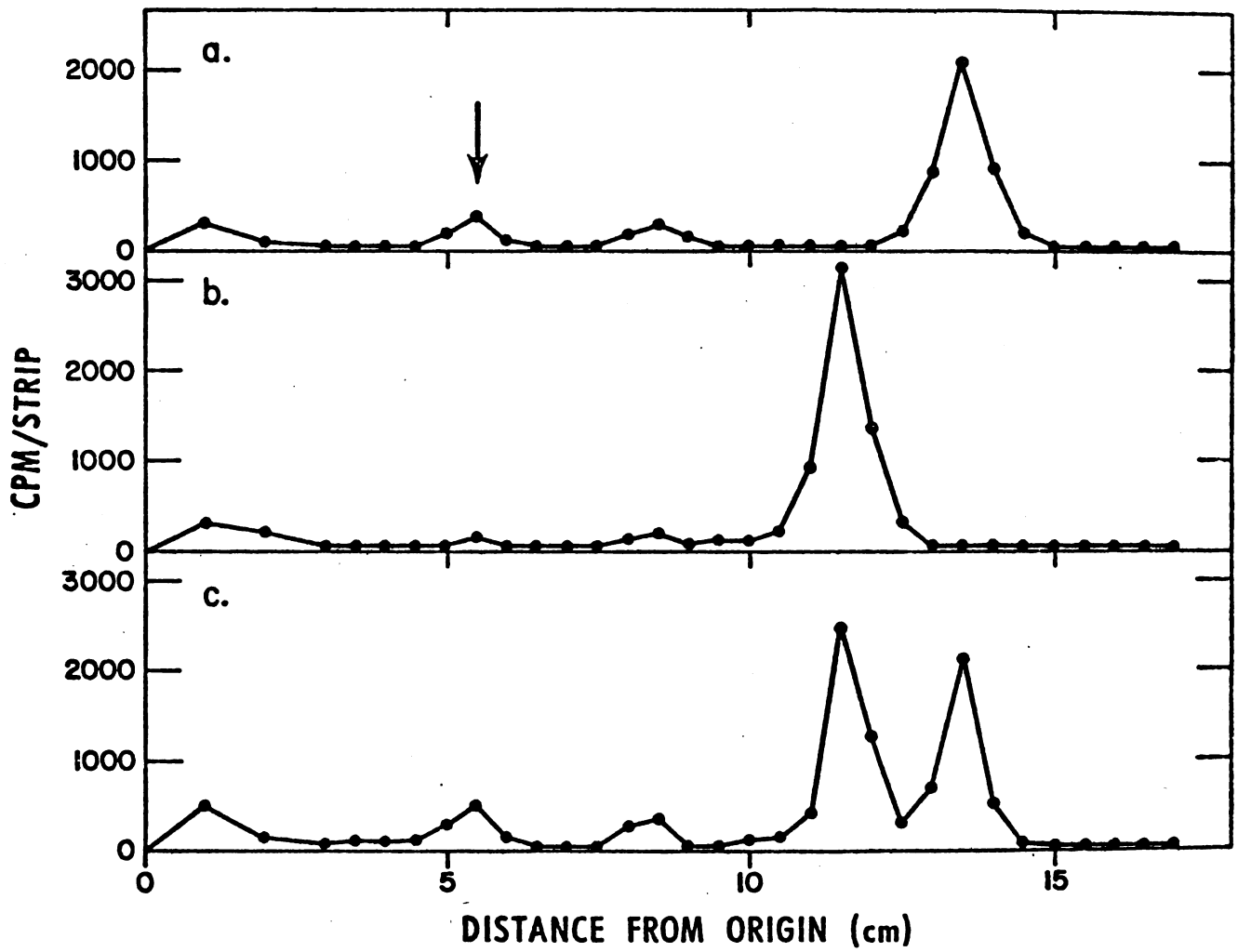
bromoacetylated. The remainder was unreacted [^{3}H]Phe-tRNA as determined by Cu^{2+} hydrolysis (Schfield and Zamecnik, 1968). As controls, N-acetyl[^{3}H]Phe-tRNA^{ox-red} and N-acetyl-[^{3}H]Phe-tRNA^{cont} were made in an exactly comparable way using N-acetylsuccinimide.

Because of the low efficiency of incorporation of these derivatives when used as ribosome affinity labels, it was necessary to determine whether any adenosine^{ox-red} at the 3' terminal end of tRNA^{ox-red} was replaced by adenosine during the extended charging reaction required at this low phenylalanine concentration. To this end, bromoacetyl [^{3}H]Phe-adenosine^{ox-red} and bromoacetyl[^{3}H]Phe-adenosine^{cont} were obtained by treatment of the respective BrAc[^{3}H]Phe-tRNAs with pancreatic ribonuclease. They were analyzed by TLC on silica gel sheets in butanol:ethanol:water (4:1:5) at 4° C (Figure 9). The following R_f values were obtained: BrAc[^{3}H]Phe-adenosine^{ox-red} 0.64; BrAc[^{3}H]Phe-adenosine^{cont} 0.76; Phenylalanine 0.31. No (<0.8%) BrAc[^{3}H]Phe-adenosine was detected in the oxidized-reduced sample. The small peak at R_f 0.47 is probably bromoacetyl-phenylalanine.

Figure 7

Characterization of the reaction products formed by pancreatic RNase digestion of Bromoacetyl-Phe-$tRNA^{ox-red}$ and Bromoacetyl-Phe-$tRNA^{cont}$

22,500 cpm of Bromoacetyl[^{3}H]Phe-$tRNA^{ox-red}$ and 18,300 cpm of Bromoacetyl[^{3}H]Phe-$tRNA^{cont}$ prepared as described in Chapter II.B.9., in 15 µl of 1 mM sodium acetate, pH 5.5 were treated with 5 µg of pancreatic RNase and incubated for 15 minutes at 30° C. 6 µl of the mixture from the control sample (Panel a), 5 µl from the oxidized-reduced sample (Panel b), and the same amounts of both samples (Panel c) were applied to a silica gel thin layer chromatography sheet (with fluorescent indicator; Eastman Kodak Co.) and chromatographed in the upper phase of the solvent system, n-butanol:ethanol:water (4:1:5) at 4° C. The chromatogram was dried, cut into strips and counted under toluene scintillation fluid. The arrow shows the position of free phenylalanine.


a.
b.
c.
2000
1000
0
3000
2000
1000
0
3000
2000
1000
0
CPM/STRIP
0
5
10
15
DISTANCE FROM ORIGIN (cm)

10. Chemical synthesis of Ado(2',3'Phe) and Ado^{ox-red}(2,3'Phe)

The mixed 2' and 3' isomers of Ado(Phe) and Ado^{ox-red}(Phe) were obtained by condensing 5'-O-trityladenosine and 5'-O-trityl-adenosine^{ox-red}, respectively, with N-tertbutyloxcarbonyl-L-phenylalanine (t-BOC-Phe) in the presence of dicyclohexylcarbodiimide (DCC), followed by removal of all protecting groups by treatment with trifluoroacetic acid (Figure 8) (Chladek et al., 1970).

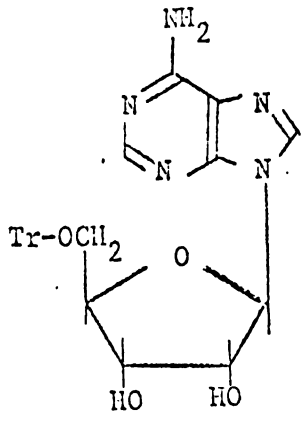
a. Trityl-adenosine and trityl-adenosine^{ox-red}

Oxidation of 5'-O-trityladenosine (obtained from Schwarz-Mann) with the resultant formation of the 2', 3'-dialdehyde in the pentose moiety was achieved by sodium periodate treatment at pH 5.5 (Figure 8, step 1). The subsequent reduction of the dialdehyde by sodium borohydride in alkaline medium resulted in diol formation (Figure 8, step 2).

300 mg (0.59 mmoles) of 5'-trityladenosine was dissolved by heating at 100° in 32 ml of pyridine-acetate, pH 5.5. 6 ml of 0.5 M sodium periodate (3 mmoles) and 27 ml of water were added and the mixture was incubated in the dark at room temperature. After 30 minutes, 3 mmoles of solid sodium borohydride was then added to the mixture gradually and incubated for one hour in the dark at room temperature. At 30 minutes, an additional 1.12 gm sodium borohydride and 10 ml of water to dissolve the borohydride were added and incubation was continued. Overheating of the reaction mixture due to borohydride addition was prevented by cooling the flask in ice. After one hour 5'-trityladenosine^{ox-red} was precipitated by the addition of five volumes of cold water at 0° for 3 hours. The resultant precipitate was collected and washed exhaustively with water to remove all borohydride. It was dried over phosphorus pentoxide for three days. The final yield was 80%.

Figure 8

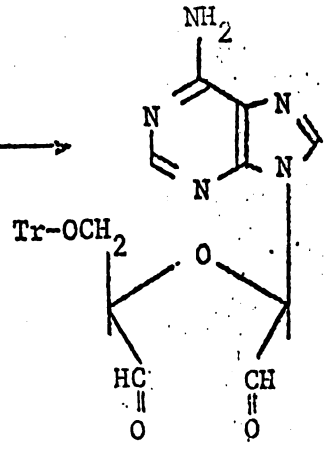
Chemical Synthesis of 2', (3')-O-Phenylalanyl derivative of oxidized-reduced adenosine



Trityl-Adenosine

$NaIO_4$

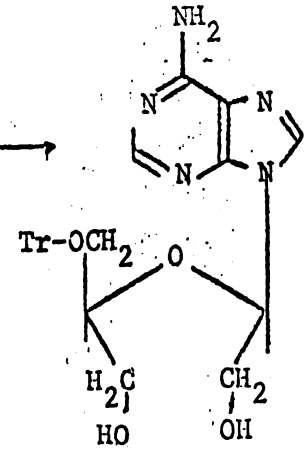
1



Oxidized
Trityl-Adenosine

$NaBH_4$

2

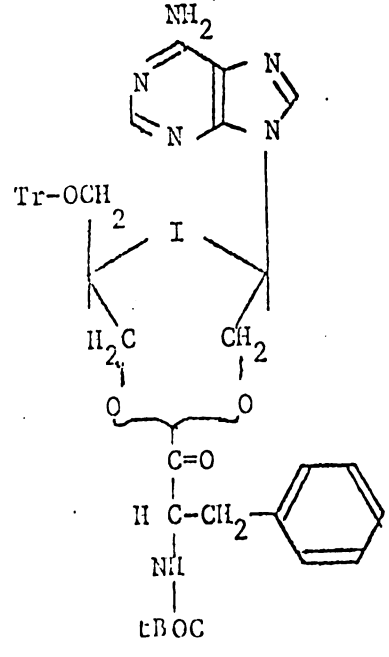


Oxidized-reduced
Trityl-Adenosine

tBOC-Phenylalanine

DCC

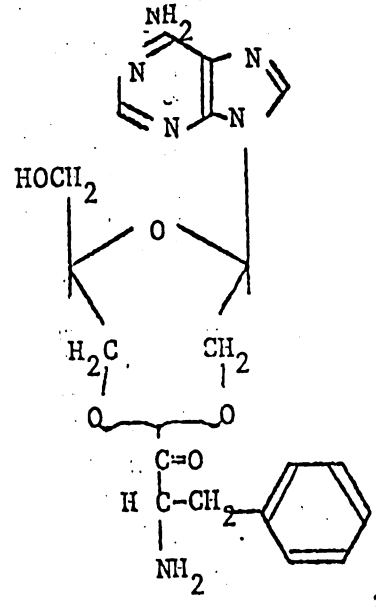
3



2,(3')-tBOC-O-phenylalanyl
derivative of oxidized-reduced
Trityl-Adenosine

TFA

4



2,(3')-O-phenylalanyl
derivative of oxidized-reduced
Adenosine

Tests for the extent of oxidation and of reduction were performed by TLC both before and after removal of the trityl group. For the removal of the trityl group anhydrous TFA was added to the solid compound (100 µl/mg) and incubated at 25° C for 20 minutes. TFA was removed under vacuum at 25° C, the residue washed three times with diethylether and dissolved in pyridine.

The oxidized 5'-O-trityladenosine ($TrAdo^{ox}$), the oxidized-reduced 5'-O-trityladenosine ($TrAdo^{ox-red}$), and their detritylated products gave single spots on TLC in all solvent systems tested (Table I). Consequently, under these conditions practically all of the 5'-O-trityladenosine was oxidized and subsequently reduced.

b. TrAdo (2',3'Phe) and $TrAdo^{ox-red}$ (2',3'Phe)

A solution of DCC (130 mg; 0.6 mmoles) in 2 ml of pyridine (dried over molecular sieves, type 4A beads) at 0° C was added to a solution of dry TrAdo (230 mg; 0.45 mmoles) or $TrAdo^{ox-red}$ (200 mg; 0.391 mmoles) dissolved in 10 ml of pyridine at 0° C. To this mixture was added in 3 ml of pyridine an equimolar amount of t-BOC-phe; 119 mg (0.45 mmoles) to the TrAdo solution or 125 mg (0.47 mmoles with radioactive specific activity of 0.082 uCi/µmole) to the $TrAdo^{ox-red}$. Radioactive t-BOC[^{14}C]-phe was used in the condensation with $TrAdo^{ox-red}$ primarily for convenient localization and isolation of the product (Figure 8, step 3). The mixture was maintained for one hour at 0° C and for an additional 48 hours at room temperature. The extent of reaction was checked by TLC on silica gel with benzene:methanol (95:5) or methylene chloride:methanol (95:5)as solvent. In these solvents, the condensation products moved faster than the reactants TrAdo or $TrAdo^{ox-red}$ (Table II). After the reaction was complete, a small

Table I

Characterization of Ado^{ox}, Ado^{ox-red} and Their Trityl Derivatives

Compound	R_f in System		
	A	B	C
TrAdo	0.22	-	-
$TrAdo^{ox}$	0.37	-	-
$TrAdo^{ox-red}$	0.31	-	-
Ado	0.06	0.55	0.27
Ado^{ox}	-	0.66	0.37
Ado^{ox-red}	-	0.59	0.30

System

A: Benzene/methanol = 95/5 on silica gel (Eastman)

B: Butanol/isopropanol/ammonia/water = 3/3/1/1 on Avicell cellulose (Merck)

C: Methylene chloride/methanol = 90/10 on silica gel

Samples were prepared as described in Chapter II.B.10.a. and located by viewing under ultraviolet light.

piece of ice was added (to destroy the residual DCC) followed by 1 ml of light petroleum added. The precipitate of DCC was filtered off and the filter paper washed with 10 ml of pyridine. The combined filtrate was evaporated to dryness under reduced pressure at room temperature and the residue dissolved in a minimum volume of methylene chloride. Purification of the reaction products was achieved by TLC on preparative (2 mm thick) silica gel plates (with fluorescent indicator) which were pre-washed with methylene chloride:methanol (20:80) to remove yellow-colored materials from the silica gel. 20-40 mg of the reaction mixture was applied to a 20 x 20 cm plate and chromatographed in benzene: methanol (95:5) with repeated development (around 10 runs) until clear separation was achieved as viewed under ultraviolet light. The $TrAdo^{ox-red}$ reaction mixture gave three bands b_2, b_3, b_4 (at 4, 10, and 15 cm respectively from the origin), which moved faster than the reactants (b_1 moved 1 cm). The mobility of these bands in this case does not relate to their true R_f values. The edge of the silica gel around the UV-absorbing bands were carefully cut out as a narrow channel and the plate developed again at 90 degrees until the bands concentrated into a small area. The silica gel containing the ultraviolet-absorbing area were then scraped out and packed separately into a small dry column. The UV-absorbing material were then eluted with ten volumes of methylene chloride:methanol (70:30), the solvent evaporated under reduced pressure, the residue dissolved in a minimum volume of methylene chloride and analyzed by TLC on silica gel sheets with benzene:methanol (95:5) as developer. The two faster moving bands b_3 and b_4 each gave one major UV-absorbing spot but only the UV-spot from b_3 contained radioactivity. However, both b_3 and b_4 were pyridine derivatives as judged by their

ultraviolet absorption spectra in ethanol. The slower moving band b_2 gave two UV-absorbing spots; the slower moving spot had no radioactivity associated with it and comigrated with trityladenosine^{ox-red} whereas the faster moving spot contained radioactivity. The entire material from band b_2 was re-chromatographed on a preparative silica gel plate in methylene chloride:methanol (95:5) and the desired fast moving UV-absorbing radioactive band was eluted as above. Subsequent analysis in benzene:methanol (95:5) as above showed a single UV-absorbing spot co-incident with the single radioactive spot. The eluate possessed a typical ultraviolet absorption spectrum in ethanol (Chladek et al., 1970) for Ado(Phe) after the removal of protecting groups as mentioned below. The final yield was 30%.

During the synthesis of TrAdo(Phe), also three bands B_2, B_3, B_4 (at 3, 5.5 and 17.5 cm respectively) were obtained. B_4 was identified as a pyridine-product as described above. Bands B_2 and B_3 were re-chromatographed as for b_3 above and gave a typical adenosine ultraviolet absorption spectrum in ethanol after removing the protecting groups as described below. The final yields were 33% and 13% respectively. These values agree with reported yields (Chladek et al., 1970).

The removal of both of the protecting groups (trityl and t-BOC) from b_3, B_2, and B_3 was achieved by TFA treatment as described in section B.10.a. (Figure 8, step 4).

c. Characterization of the products

The final condensation products were characterized before and after removal of the protecting groups (trityl and t-BOC) on TLC in four solvent systems (Table II) and by paper chromatography and high voltage electrophoresis (Table III) which show clearly that it

Table II

Thin-layer Chromatography of Starting Compounds, Final Compounds and Standards

Compound	R_f in Systems A	B	C	D
TrAdo^{ox-red}(tBOCPhe) (band b_2)	0.38	0.90	0.71	
TrAdo^{ox-red}	0.31	0.72	0.68	
Ado^{ox-red}(Phe)	0.04	0.18	0.55	
Ado^{ox-red}(Phe) Alkali treated	-	0.12	0.53	
Ado^{ox-red}	-	0.12	0.53	
TrAdo(tBOCPhe) (band B_2)	0.30	0.87	0.70	
TrAdo	0.22	0.70	0.67	
Ado(Phe)	-	0.17	0.57	0.51
Ado(Phe) Alkali treated	-	0.13	0.49	0.42
TrAdo $(tBOCPhe)_2$ (band B_3)	0.39	0.89	0.71	
$Ado(phe)_2$	-	0.34	0.57	0.52
Ado	0.06	0.12	0.50	0.41
Ad	0.02	0.22	0.53	0.55

Solvent system:

A = Benzene/methanol = 95/5
B = Methylene chloride/methanol = 95/5
C = n-Butanol/acetic acid/water = 5/2/3
D = n-Butanol/ethanol/water = 4/1/5
(Upper phase)

Samples were prepared as described in Chapter II.B.10.a. and b. and located by viewing under ultraviolet light after chromatography on silica gel sheets (Eastman).

Table III

Paper Chromatography and Electrophoresis of Ado(Phe)

$Ado(Phe)_2$ and $Ado^{ox-red}(Phe)$

Compounds	Chromatography(R_f)		Electrophoresis (cm)	
	UV	Ninhydrin	UV	Ninhydrin
$Ado^{ox-red}(Phe)$	0.54	-	16.9	-
$Ado^{ox-red}(Phe)$, alkali treated	0.34	0.44	11.9	5.5
Ado^{ox-red}	0.35	-	12.2	-
Ado(Phe)	0.58	-	17.0	-
Ado(Phe), alkali treated	0.34	0.42	11.8	5.1
$Ado(Phe)_2$	0.53	-	19.9	-
$Ado(Phe)_2$, alkali treated	0.33	0.42	11.3	5.3
Ado	0.34	-	12	-
Ad	0.45	-	24	-
Phe	-	0.41	-	5.5

Samples were prepared as described in Section B.10.a. and b. Chromatography was done on Whatman No. 3 MM paper in butanol:ethanol: water (4:1:5 upper phase). After the location of the samples under UV light, the chromatogram was sprayed with 0.2% Ninhydrin (in acetone plus 1% acetic acid).

Electrophoresis was carried out on a 50 x 10 cm Whatman No. 1 paper in 0.05 M sodium citrate buffer, pH 3.4 for 3 hours at 3000 volts on a Gilson high voltage electrophoretor. Samples were detected as described above. All compounds tested moved toward the cathode.

contained Ado^{ox-red} (or Ado) and phenylalanine and that the phenylalanine was linked by an alkali-sensitive ester bond. For the use of these compounds in peptidyl transferase assay, it was important to determine the stoichiometry between Ado^{ox-red} (or Ado) and the phenylalanine. This was achieved as follows.

The ratio of nucleoside/Phe was determined after hydrolysis of the ester linkage with 0.5 N sodium hydroxide for 20 minutes at 37° C. The nucleoside was estimated by its absorption at 257 nm in 0.01 N HCl, using an extinction coefficient of 1.51×10^{-3} for Ado (Morell and Bock, 1954) and assuming the same value for Ado^{ox-red}. Phenylalanine was quantitated on an amino acid analyzer using the fluorescamine reaction as described by Stein et al., (1973). As shown in Table IV, it is evident that the band b_2 was mono-substituted while bands B_2 and B_3 were mono-and disubstituted respectively. Since the phenylalanine in Ado^{ox-red}(Phe) was radioactively labelled (1.6×10^5 cpm/μmole), the amount of phenylalanine was also determined by the content of radioactivity. The amount of phenylalanine present in Ado^{ox-red}(Phe) determined by both methods were in good agreement (Table IV). Also shown in the same table are the UV-absorption characteristics of the synthesized compounds which agree with the reported values for Ado(Phe) and $Ado(Phe)_2$ (Chadlek et al., 1970).

Since the purpose of these syntheses was to compare the acceptor activity of $Phe\text{-}tRNA^{ox-red}$ (in which the 3'-terminal adenosine bearing the phenylalanine is oxidized-reduced) in peptide transfer reaction, the chemically synthesized Ado^{ox-red}(Phe) was compared with the Ado^{ox-red}(Phe) derived from $Phe\text{-}tRNA^{ox-red}$. As shown in Figure 5, both the chemically synthesized and the biologically prepared Ado^{ox-red}(Phe) have the same R_f on TLC. This presents an additional confirmation that

the synthetic $Ado^{ox-red}(Phe)$ was structurally the same as $Ado^{ox-red}(Phe)$ present at the 3'-end of $Phe-tRNA^{ox-red}$.

In addition, this analysis as well as the analysis given in Table I can be used to place an upper limit to the amount of Ado(Phe) that could be contaminating the $Ado^{ox-red}(Phe)$ preparation. From Figure 5, 2% could be Ado(Phe), while TLC analysis of $TrAdo^{ox-red}$ (Table I) showed no detectable (< 0.5%) Tr-Ado in $TrAdo^{ox-red}$.

Table IV

Ultraviolet Absorption spectrum and Ado/phe Ratio in $Ado^{ox-red}(Phe)$, Ado(Phe), and $Ado(Phe)_2$

Compound	UV Absorption			Ratio
	λ-max (0.01 M HCl)	250/260	280/260	Ado/Phe
$Ado^{ox-red}(Phe)$	256	0.97	0.17	0.85, 0.87*
Ado(phe)	256	0.96	0.17	0.92
$Ado(phe)_2$	255	0.95	0.17	1.70

* Phenylalanine estimation by radioactivity.

Adenosine and phenylalanine were estimated as described in text.

Samples were prepared as described in Section B.10.b.

C. Preparation of Protein Synthesizing Components

Preparation of aminoacyl-tRNA synthetases, amino acid incorporating enzymes and washed ribosomes from E. coli

Cell Extract: Either commercially available or freshly grown E. coli Q13 log-phase frozen cells were used for preparation of aminoacyl-tRNA synthetases, enzymes for incorporation of amino acids into polypeptides, and washed ribosomes. 15 gm E. coli frozen paste was allowed to thaw, and then homogenized at low speed without foaming in a Waring blendor with 40 ml of 0.01 M Tris-HCl, pH 8.0, 0.01 M $MgCl_2$, 10% glycerol. The cells were lysed by passing the cell homogenate through a French Pressure Cell at 8,000 psi. RNase-free-DNase, 2.5 µg/ml, was added and the extract incubated for 5 minutes at 4° C. This was required if the extract was viscous. The suspension was centrifuged at 20,000 x g and 30,000 x g for 20 and 30 minutes respectively (to remove cell debris) in SS-34 rotor (Sorvall). The supernatant (S-30) was centrifuged at 50,000 rpm in a Spinco Type 60 Ti rotor for 2 hours at 0° C. The top 3/4 of the resultant supernatant was carefully aspirated and used (S-100) for the preparation of aminoacyl-tRNA synthetases and amino acid incorporating enzymes. The bottom viscous 1/4 layer of the supernatant was discarded and the pellet was used for preparing washed ribosomes. All operations were carried out at 4° C.

1. Preparation of mixed aminoacyl-tRNA synthetases from S-100

The procedure is identical to the method described by Muench and Berg (1966). The high speed supernatant (S-100) was applied to a DEAE-cellulose column (2 x 40 cm) equilibrated with 0.02 M potassium phosphate buffer, pH 7.5, 0.02 mercaptoethanol, 0.001 M $MgCl_2$ and 10% glycerol (v/v). The column was washed with the same buffer at a flow rate of 60 ml per hour. After the A_{280} was down to 0.07 (at about 1 liter), the elution buffer was changed to 0.25 M potassium phosphate, pH 6.5, 0.02 M mercaptoethanol, 0.001 M $MgCl_2$ and 10% glycerol (v/v) to elute the enzyme. Fractions of 5 ml were collected at a flow rate of 60 ml per hour. Absorbance at 280 mμ was recorded and the peak tubes were pooled. The pooled enzyme was dialyzed against 6 liters of 0.01 M potassium phosphate, pH 6.9, 0.02 M mercaptoethanol, 10% glycerol (v/v) and 15% polyethylene glycol at 2° C until the dialysate was 1/5th of the pooled volume. It took about 20 hours. To the concentrated enzyme, 0.8 volume of glycerol was added and gently mixed and stored at -30° C. All operations were conducted at 4° C unless otherwise stated. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the reference.

2. Preparation of crude amino acid incorporating enzymes from E. coli

Amino acid incorporating enzymes were prepared by the method of Mathaei and Nirenberg (1961). The higher speed supernatant (S-100) was dialyzed 4-6 hours at 2-4° C against 10 mM Tris-acetate pH 7.8, 10 mM magnesium acetate, 60 mM KCl, 6 mM mercaptoethanol and 10% glycerol. 0.8 volume of glycerol was added to the dialysate. It was divided into small portions and stored at -30° C. Protein concentration was determined by the method of Lowry (1951) with bovine serum albumin as the reference.

3. Preparation of E. coli ribosomes and ribosomal subunits

1x washed ribosomes.

The method is essentially that of Mathaei and Nirenberg (1961). The high speed pellet was suspended in 10 mM Tris-acetate, pH 7.8, 10 mM MgOAc, 60 mM KCl, 6 mM mercaptoethanol, and 10% glycerol and centrifuged at 60,000 rpm for 1.5 hours in the Spinco 65 Ti rotor. The pellet was suspended in the cold buffer and spun at 30,000 x g for 30 minutes. The resultant supernate was again centrifuged at 60,000 rpm for 1.5 hours and the final ribosomal pellet suspended in the above buffer. Unless otherwise stated, these ribosomes were used for the phenylalanine incorporating studies and for making polyphenylalanine charged ribosomes.

3x NH_4Cl-washed ribosomes.

Washed ribosomes used in peptide bond formation studies were prepared from the high speed pellet according to the method described by Zamir et al. (1972). The pellet was suspended in 10 mM Tris-acetate, pH 7.4, 10 mM $MgCl_2$, 500 mM NH_4Cl, and centrifuged at 50,000 rpm for

2 hours. The supernatant was discarded and washing of the pellet was repeated three times in the same buffer with alternate low speed run. The final ribosomal pellet was suspended in 10 mM Tris-acetate, pH 7.4, 50 mM NH_4Cl, 10 mM $MgCl_2$ and stored in small aliquots at -170° C.

Ribosomal Subunits

For the isolation of 50S and 30S subunits, the procedure of Miskin et al. (1970) was followed. The NH_4Cl-washed 70S ribosomes obtained above were dissociated by a 24-hr dialysis against 10 mM Tris-acetate, 1 mM $Mg(OAc)_2$, 100 mM NH_4Cl. Five ml of the ribosomal suspension (20 mg/ml), was layered over 25 ml of a linear 5-20% sucrose gradients in the same buffer and centrifuged at 2°C for 15 hours at 25,000 rpm (Spinco rotor SW 25.1). Analysis of gradients was performed by puncturing the bottom of the tube followed by pumping 40% sucrose (w/v) in the above buffer into the bottom of the tube with a syringe pump at a flow rate of 1 ml per min. Gradients were analyzed with a flow cell (Instrument Specialities Co.) seated directly on top of the gradient tube and coupled to the optics of a Gilford model 2400 recording spectrophotometer. Absorbance was recorded at 260 nm. 50S and 30S subunits were distinctly separated from each other. Fractions were pooled and dialyzed against 10 mM Tris-acetate, pH 7.4, 1 mM $Mg(OAc)_2$, 100 mM NH_4Cl for 10 hours and stored in liquid N_2. Unless otherwise stated, these 50S subunits were used for peptidyl transferase activity studies, and the 30S subunits were used for the enzymatic binding of NAcPhe-tRNA in the presence of crude initiation factors. Activation of 70S and 50S ribosomes for peptidyl transferase activity was routinely performed according to the method of Miskin et al. (1970). Ribosomes were incubated in 50 mM Tris-acetate, pH 7.4, 500 mM NH_4Cl,

10 mM $Mg(OAc)_2$ for 15 minutes at 40°. They were chilled in ice and diluted to the desired salt concentration.

Ribosomes for Affinity Labelling

For the ribosomal binding of BrAc[^{3}H]Phe-tRNA, 70S ribosomes were prepared from mid-logarithmic phase E. coli Q13 by the method of Traub et al. (1971). The following buffers were used. A (10 mM Tris-HCl, pH 7.4, 50 mM NH_4Cl, 6 mM 2-mercaptoethanol, 10 mM $MgCl_2$; B (Same as A except 0.25 mM $MgCl_2$): C (same as A except 1.0 μM $MgCl_2$ and no 2-mercaptoethanol).

The crude extract, free of cell debri, was centrifuged at 30,000 rpm for 6 hours in a Spinco rotor 60 Ti. The ribosomal pellet was suspended in about 15 ml of buffer A and the insoluble material was sedimented by centrifuging at 18,000 rpm for 10 min in the SS-34 rotor of the Sorvall centrifuge. 10 ml portions of the ribosome solution were layered on top of 28 ml of 30% sucrose in solution A and centrifuged for 18 hours at 30,000 rpm in rotor 60 T. The pellet was resuspended and the last step was repeated. The pelleted material was again dissolved in solution A and the resulting solution was freed from slight turbidity by low speed centrifugation.

30S and 50S ribosomal subunits were prepared from these 70S ribosomes by dialysis against buffer B for 48 hours at 4° C, and were separated on 10-30%, 38-ml sucrose gradients as described above. Unfolded ribosomes were prepared by dialysis of 70S particles against buffer C for 48 hours at 4° C.

4. Preparation of ribosomes containing polypeptide chains.

Ribosomes containing nascent polyphenylalanine chains were prepared according to the method described by Maden et al. (1968). The reaction mixture contained, in a final volume of 1 ml, 181 A_{260} units of ribosomes, 20 A_{260} units of unfractionated tRNA, 6.6 A_{280} units of the amino acid incorporating enzyme fraction (section 2), 1.0 mM phosphoenolpyruvate, 13 µg of phosphoenolpyruvate kinase, 50 mM tris-acetate, pH 7.4, 100 mM NH_4Cl, 1 mM glutathione, 10 mM $Mg(OAc)_2$, 0.5 mg of poly U, 0.5 mM GTP and 0.05 mM [^{14}C]Phenylalanine (10 mCi/mmole). The mixture was incubated at 37° C for 20 minutes and chilled in ice. After dilution to 10.5 ml with cold Wash Buffer (10 mM Tris-acetate, pH 7.4, 10 mM magnesium acetate, 50 mM NH_4Cl), it was centrifuged in the Spinco 65 Ti rotor at 40,000 rpm for 2 hours at 0° C (high speed). The resultant pellet was suspended in 6 ml of Wash Buffer and centrifuged at 15,000 x g for 10 minutes (low speed). The supernatant from the low speed run was again diluted to 105 ml and centrifuged at high speed. After a second cycle of low and high speed centrifugation, the ribosomal pellet was suspended in 1 ml of cold 10 mM Tris-acetate, pH 7.4, and 10 mM magnesium acetate and stored at 0° C. These ribosomes were designated as "labelled ribosomes." Radioactivity in the washings was measured directly after each high speed centrifugation step to ensure complete removal of free [^{14}C]phenylalanine. Approximately 10 molecules of phenylalanine were incorporated in polypeptide per molecule of ribosome if all of the ribosomes were active.

5. Preparation of yeast aminoacyl-tRNA synthetases

The method was similar to that described by Lindhal et al. (1967). 100 gm of fresh yeast cells (Red Star Corp.), 200 gm of acid

washed glass beads (0.2 mm) from Schwarz-Mann and 20 ml of 0.05 M Tris-HCl, pH 8.0, 0.01 M $MgCl_2$ and 0.005 M dithioerythretol were placed in a 400 ml metal Omni-mixer container. The container was cooled in an ice-water bath and the contents were homogenized for 0.5 minutes at full speed. The contents were mixed with a clean spatula and homogenization continued for an additional 0.5 minutes. Cells were checked for proper mixing and the container allowed to cool for at least 1 minute. Alternate homogenization for 0.5 minutes and cooling for 1 minute were carried out for a total of 10 minutes. 100 ml of cold buffer (0.02 M Tris-HCl, pH 8.0, 0.004 M $MgCl_2$, 0.002 DTT) was then added to the cell slurry and it was mixed at low speed for 7 minutes. The contents were spun at 15,000 rpm (27,000 x g) in a Sorvall SS-1 rotor for 15 minutes and the supernatant (125 ml) was centrifuged at 50,000 rpm in a 60 Ti Spinco rotor for 2 hours at 2° C. The final supernatant (100 ml) was concentrated by dialysis against 4 liters of 15% polyethylene glycol in the above buffer. The final dialysate was centrifuged at 60,000 rpm for 2 hours to remove residual ribosomes. The supernatant was loaded onto a Sephadex G-25 column (2.5 x 36), equilibrated with 2 mM Tris-HCl, pH 8.0, 4 mM $MgCl_2$ and 0.2 mM mercaptoethanol and eluted. Absorbance at 280 nm was recorded and the first protein peak was pooled, made 50% by glycerol, and stored at -30° C. The enzyme was stable at -30° C at least for one year.

6. Preparation of Crude Initiation Factors

Crude initiation factors were prepared by a modification of the procedure of Hershey and Thach (1967) as described by Weissbach et al. ((1968). 250 gm of midlog E. coli B cells, obtained from Grain

Processing Corp., were suspended in 250 ml of a solution containing 10 mM $Mg(OAc)_2$, 50 mM NH_4Cl and 12 mM β-mercaptoethanol. The cells were disrupted in a French pressure cell (8,000 psi) and 4 μg of RNase-free DNase were added per ml of suspension. After 5 minutes at 4° C, the suspension was centrifuged at 30,000 x g for 30 minutes. The top 3/4 of the supernatant fraction was removed and centrifuged at 200,000 x g for 3 hours at 4° C in a 60 Ti Spinco rotor. The pellet was suspended in 180 ml of 40 mM Tris-acetate buffer, pH 7.4, containing 1 mM $MgCl_2$ and 1 M NH_4Cl. This suspension was maintained at 4° C overnight and centrifuged at 30,000 x g for 30 min to remove a brown contaminating material. The 30,000 x g supernatant fraction was then centrifuged at 200,000 x g for 3 hours and the supernatant solution was used as the source of initiation factors. It was stored at -170° C.

7. Preparation of E. coli Polysomes

Polyribosomes were prepared according to the method described by Pestka and Hintikka (1971). E. coli Q13 was grown at 37° C at an absorbance at 650 nm of 0.5 in 5 liters of medium containing 50 gm glucose, 25 gm tryptone, 25 gm NaCl, 25 gm yeast extract and 250 μg vitamin B_1. Under these conditions, cells were still in the log phase. The cells were rapidly chilled to 0-5° within 10 seconds by swirling the culture in a stainless steel beaker surrounded by a dry ice-acetone bath. Cells were collected by centrifuging the cell suspension at 10,000 rpm for 10 minutes in the GSA rotor of a Sorvall RC2B centrifuge. They were resuspended in 70 ml of 25% (w/v) sucrose in 0.04 Tris-chloride, pH 8.1 and 49 mg of freshly prepared lysozyme in 7.7 ml of 0.04 M Tris-chloride, pH 8.0, was added. To start the action of lysozyme, 3.8 ml of 0.1 M Na_2 EDTA was then added. The concentrated cell suspension was kept in an ice bath

for 90 seconds with occasional shaking. The action of lysozyme was stopped by adding 0.93 ml of 1 M $MgSO_4$ to the mixture. The protoplast suspension was sedimented at 10,000 x g for 5 minutes in the SS-34 rotor of the Sorvall centrifuge. The supernatant portion was discarded carefully. The pellet was resuspended in 15 ml of lysing medium containing 0.5% (w/v) Brij 58, 0.05 M NH_4Cl, 0.005 M $MgSO_4$, 0.01 M Tris-chloride, pH 7.5, by stirring with a glass rod. 0.15 mg DNase in 0.03 ml was added to the lysed cells. The suspension was kept in lysing medium for 10 minutes at 0-5° C. The viscous lysate was then centrifuged for 10 minutes in the SS-34 rotor at 10,000 x g. The supernatant was gently aspirated and the pellet discarded. The final extract contained 230 A_{260} units per ml. Since polysomes break down in cells at 0-5° C, but less so in cell lysate, care must be taken to work fast up to the lysing step. Rapid chilling is important because the yield of polysomes is drastically reduced if chilling takes longer than 15-20 seconds. Polysomes prepared by the above method were heavily contaminated with nucleases, supernatant fraction and free ribosomal particles. They were removed by sedimenting polysome extracts through a 15-35% sucrose gradient containing 0.005 M Tris-acetate, pH 7.1, 0.05 $MgCl_2$ and 0.1 M KCl. 2 ml samples were layered over an 8 ml gradient and centrifuged at 100,000 x g for 3 hours in the 60 Ti Spinco rotor to pellet the polysomes. Washing was repeated three times to obtain polysomes completely free of nuclease activity. The absence of nuclease activity was tested by the incubation of 19mer[^{14}C]Tyr (section B.3.a.) or 13mer[^{14}C]Val (Krauskopf et al., 1972) fragments with the polysome preparation at 37° C for 8 minutes. All of the input radioactivity was recovered as TCA-precipitable counts. By contrast, unwashed poly-

somes were rendered 100% acid soluble in less than one minute. These purified polysomes behaved almost identically to the crude polysome extracts with respect to peptidyl-puromycin synthesis (Pestka, 1972). Polysomes were finally suspended in 0.005 M Tris-acetate, pH 7.1, 0.05 KCl and 0.005 $MgCl_2$ and stored at -170° C.

D. Methods

1. Assay for Peptide Bond Formation on Washed E. coli Ribosomes

a. Assay for N-acetyl[^{3}H]Phenylalanyl-puromycin formation

The assay for the formation of N-acetyl-Phe-puromycin on 70S ribosomes in the presence of poly U and the absence of alcohol was a modification of the ethyl acetate extraction method of Leder and Bursztyn (1966) as described by Weissbach et al., (1968) and Rychlik et al. (1969).

100 µl of standard incubations contained 0.05 M Tris-acetate, pH 7.4, 0.1 M NH_4Cl, 0.08 M KCl, 0.02 M $MgCl_2$, 0.01 M DTT, 0.1 mg/ml poly U, 2.4 A_{260} units of washed ribosomes, 500 µM neutralized puromycin and different amounts of the donor substrate N-acetyl-[^{14}C]phe-tRNA (100 cpm/pmole) as shown for individual experiments. Incubations were at 35° C for the time indicated in the figure legends. Reactions were terminated by adding 0.1 ml of saturated $MgSO_4$ in 0.1 M potassium acetate, pH 5.2, (Maden and Monro, 1968) followed by 3 ml of ethyl-acetate to extract the N-acetyl-Phenylalanyl-puromycin formed. The contents of the tube were mixed for 45 seconds on a vortex mixer, centrifuged briefly at low speed and 2 ml of the upper ethyl-acetate phase was pipetted into 15 ml of Bray's solution (Bray, 1960), and the radioactivity determined. The amount of N-acetyl-Phe residue transferred from N-acetyl-Phe-tRNA to the acceptor was determined as the difference in radioactivity extracted into ethyl acetate upon incubation with and without acceptor. The values obtained, corrected for 3 ml of ethyl acetate, were expressed either as

percentage of N-acetyl-Phe-tRNA used in the experiment or as pmole product formed. Under these conditions, ethyl acetate extraction of N-acetyl-Phe-puromycin was reported to be over 95% (Maden and Monro, 1970).

b. Assay for N-acetyl-Phe-puromycin formation on 70S ribosomes or 50S ribosomal subunits by the "alcohol reaction"

The transfer of N-acetyl-Phe residue from donor to puromycin was estimated essentially as described by Miskin et al. (1970) for the reaction between fMet-tRNA or fMet-oligonucleotide with puromycin on 70S or 50S ribosomes in the presence of alcohol. Before the addition of alcohol, each reaction mixture was 0.1 ml and contained 60 mM Tris-HCl, pH 7.4, 400 mM KCl, 20 mM $Mg(OAc)_2$, 1 mM neutralized puromycin, N-acetyl-[^{14}C] or [^{3}H]Phe-tRNA or N-acetyl-Phe-oligonucleotides as indicated, and 5 A_{260} units of 50S (or 70S) ribosomes. Usually the donor substrate was added last and the reaction was started by the addition of 25 µl of methanol, although sometimes methanol was added earlier and the reaction was initiated with the addition of donor. After incubation at 22° C for up to 30 minutes as indicated, 10 µl of 10 N NaOH was added to stop the reaction and the mixture incubated at 37° C for 5 minutes to hydrolyze the methyl ester of N-acetyl-phenylalanine formed due to the presence of methanol as well as to hydrolyze the donor substrate. One ml of 1 M potassium phosphate, pH 7.0, was then added and the N-Ac-Phe-puromycin product formed was extracted with 3 ml of ethylacetate. 2 ml of the upper ethylacetate phase was counted in 15 ml of Bray's solution. Unless otherwise stated, a background value of 50-80 cpm obtained without puromycin was subtracted from values obtained with puromycin. The results

were corrected for the sample size of 3 ml and expressed as pmoles N-acetyl-phe-puromycin formed. Extraction of the product into ethylacetate was assumed to be quantitative as reported by Miskin et al (1970) for fMet-puromycin.

2. Assay for peptide bond formation on Washed polyribosomes

a. Assay for Peptidyl-[^{14}C]tyrosine formation on polysomes.

The measurement of peptidyl-[^{14}C]tyrosine formation used a modification of the procedure described by Pestka (1972) to measure peptidyl-puromycin formation with unwashed polysomes. Each 50 µl reaction mixture contained 50 mM Tris-acetate, pH 7.2; 10 mM NH_4Cl, 100 mM KCl, 5 mM $MgCl_2$, 1.0 A_{260} unit of washed polysomes and acceptor substrates as specified in the figure legends. The reaction mixture was pre-warmed for 1 minute at 24° C and polysomes were added last to start the reaction. The reaction was stopped by addition of 10 µl of 1.2 NaOH and the mixture incubated for 10 minutes at 37° C to hydrolyze product and unreacted acceptor substrate from the oligonucleotide. After addition of 10 µl of 1.2 N HCl to neutralize the alkali, peptidyl-[^{14}C]tyrosine was precipitated by addition of 3 ml of cold 10% TCA at 0° for 10 minutes. The precipitate was collected over BDWP-type millipore filters, washed five times with 2 ml of cold 5% TCA and then 8 times with ethanol at room temperature as described by Pestka (1972). The filter was dried and the radioactivity counted in toluene scintillation fluid. The blank value was obtained for <u>each</u> Tyr-oligonucleotide concentration after omitting polysomes from the complete reaction system. It was subtracted from the value obtained from incubation of the equivalent concentration of Tyr-oligonucleotide with polysomes. The saponification step was essential so as to eliminate the effect of solubility differences in TCA of Tyr-oligonucleotides differing in their chain lengths. For example, 19mer-Tyr used was insoluble in 10% TCA while tetramer-Tyr was soluble.

b. Assay for peptidyl[^{3}H]puromycin and peptidyl-AA adenosine formation on polysomes.

The conditions and components of the reaction mixtures were as described above for peptidyl-[^{14}C]tyrosine formation except that saponification with alkali was omitted. The reaction was stopped by addition of 3 ml of cold 10% TCA, the precipitate collected and washed as described above for peptidyl-[^{14}C]tyrosine formation. This procedure was used for estimating the formation of peptide product formed between peptidyl-tRNA on the polysomes and aminoacyl-adenosines such as Ado(Tyr), Ado(Phe) and Ado^{ox-red}(Phe) as well as puromycin.

3. Counting Procedure

For estimation of radioactivity, samples were placed in low background borosilicate glass vials and counted in either the Beckman LS-100 or Beckman LS-250 liquid scintillation spectrometer. In each case, the efficiency of counting was determined for the particular isotope under the same conditions as those employed in the experiment using [^{3}H]toluene or [^{14}C]toluene standards. The following procedures were used to determine the counting efficiency.

a. For the formation of N-acetyl[^{3}H]Phe-puromycin and N-acetyl[^{3}H]Phe-Phe-Ado, (Figures 12, 13, 17, 18 and 20) where the product was extractable in ethyl acetate, a [^{3}H]toluene standard was counted in 2 ml of an ethyl acetate extract of the reaction mixture in 15 ml of Bray's solution (Bray, 1960). An efficiency of 30% was obtained for [^{3}H]. For formation of N-acetyl[^{3}H]Phe-Phe-Ado^{ox-red} (Figures 17, 18, 20; see also Chapter III.3.c.) where the acceptor was weakly radioactive (Ado^{ox-red}[^{14}C]Phe; 0.018 mc/mmole but was also considerably extracted in ethyl acetate, the counting of the ethyl acetate-extractable product was done in the [^{3}H] channel with exclusion of [^{14}C]. In this case a correction due to spill over of [^{14}C] counts to the [^{3}H] channel was necessary. It was determined by counting a [^{14}C]toluene standard in 15 ml of Bray's solution in the presence of 2 ml of ethyl acetate and estimating the percentage of [^{14}C] counts in the [^{3}H] channel with respect to that in the [^{14}C] channel. An efficiency of 23.5% for [^{3}H] was obtained in the narrow [^{3}H] channel.

When the product was N-acetyl[^{3}H]phe-[^{14}C]Tyrosine (Figure 24) and counted in the [^{14}C] channel with exclusion of [^{3}H], the same procedure was adopted except that the standard used was [^{14}C]toluene. For [^{14}C], an efficiency of 92% and 44% were obtained in the [^{14}C] + [^{3}H] channel and the exclusive [^{14}C] channel respectively.

b. In the experiments of Figure 16 where product formation (CACCA-[^{3}H]Phe-[^{14}C]Phe),(soluble in TCA) was measured from the loss of precipitable [^{3}H] cpm on the millipore filter, the efficiency was calculated by counting the [^{3}H]toluene standard in 15 ml of Bray's solution in which a TCA washed millipore filter was dissolved. The efficiency was 30%.

c. When the [^{3}H] or [^{14}C] labeled reaction product was collected on BDWP-Millipore filters, dried, and counted under toluene scintillation fluid, (Peptidyl-[^{3}H]puromycin, Peptidyl[^{14}C]tyrosine, Figure 28) the [^{3}H] and [^{14}C] counting efficiencies were estimated as follows. A compound of known counting efficiency in Bray's solution, e.g. ([^{3}H]Puromycin or [^{14}C]Phenylalanine) (1-3 µl) was pipetted on a dried BDWP-Millipore filter from an experimental blank tube, and counted under 10 ml of toluene based scintillation fluid (containing 4 gm PPO, 50 mg POPOP in one liter of toluene) in which [^{3}H]puromycin or [^{14}C]-phenylalanine was not soluble. The counting efficiency was calculated with respect to its efficiency in Bray's solution. It was 25% for [^{3}H] and 78% for [^{14}C]. The same procedure was followed for the double label experiment mentioned in Table VIII and Figure 11. In this case both a [^{3}H]phenylalanine and [^{14}C]phenylalanine (the efficiencies of

which were known in Bray's solution) were counted separately on a strip of paper which was treated similarly as in the experiment under 10 ml of the toluene based scintillation fluid and the efficiencies calculated as above. Proper spill over correction was also made when [^{3}H] was counted. The same method was followed for TLC strips. An efficiency of 23% and 50% was obtained for [^{3}H] and [^{14}C] respectively in their exclusive counting channels for both paper and TLC strips.

4. Extraction efficiency

The efficiency of recovery of the reaction product by ethyl acetate extraction was nearly quantitative. It was determined when not known from the literature. In experiments of Figures 12, 13, 17 and 20 (method described in Chapter II.D.1.a.) where N-acetyl-Phe-puromycin, N-acetyl-Phe-Phe-Ado or N-acetyl-Phe-Phe-Ado^{ox-red} was the product, a recovery of over 95% in the ethyl acetate phase after one extraction was assumed. This value was reported for similar systems by Maden and Monro (1968) and Rychlik et al. (1969). In the "alcohol assay" (Method described in Chapter II.D.1.b.), an efficiency of extraction for fMet-puromycin was over 96% as found by Miskin et al. (1970). The same value was assumed for N-acetyl-Phe-puromycin formation observed in Figures 16 and 18. For N-acetyl-Phe-tyrosine (Figure 24), the extraction efficiency was determined as follows: 2 ml of the ethyl acetate extract containing the reaction product (in duplicate) was mixed with 1 ml of ethyl acetate and all the reaction components (50 µl) together with the alkali and acid used for processing of the sample as mentioned in the figure. It was back extracted and 2 ml of the separated

ethyl acetate phase was counted with 15 ml of Bray's solution. The recovery was 96% against the control (in duplicate) which consisted of 2 ml of the original ethyl acetate extract plus 1 ml of ethyl acetate.

5. Determination of K_m and V_{max}

For the determination of K_m (the Michaelis constant) and V_{max} (maximum velocity), the initial velocity (V) of product formation against the concentration of the substrate (S) was followed. Estimations of these parameters were achieved by plotting the experimental data as $\frac{S}{V}$ versus S, based on the linear transformation of the Equation,

$$\left(\frac{S}{V}\right) = \left(\frac{K_m}{V_{max}}\right) + \left(\frac{1}{V_{max}}\right) S$$

where V is the initial velocity of the reaction and S, the substrate concentration. The V_{max} was calculated from the slope of the line (slope = $\frac{1}{V_{max}}$) and the K_m from the ratio of the intercept of $\frac{S}{V}$ axis to the slope (Dowd and Riggs, 1965). The data shown in figures 21,24,25 and 28 when plotted as $\frac{1}{V}$ versus $\frac{1}{S}$ (where V and S were as defined above), gave essentially the same values for K_m and V_{max}.

CHAPTER III

EXPERIMENTAL RESULTS

1. Rationale for peptide bond synthesis using oxidized-reduced tRNA

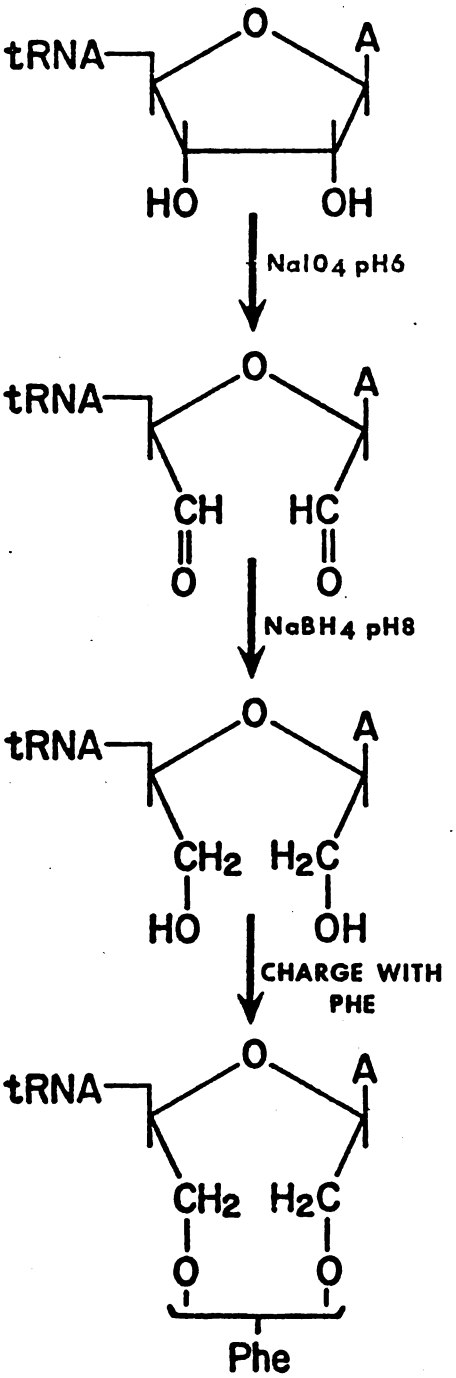
The 3'-terminal CCA sequence is present in all tRNA species so far examined and is shown to be involved in the interaction with the peptidyl transferase center during peptide bond formation (Rychlik et al., 1970). The last three bases (3'-end) of the tRNA in the donor site and the last two bases of the tRNA in the acceptor site seem to be important for peptidyl transferase activity (Monro et al., 1969). The observation that only the terminal sequences of tRNA are the substrates proper in the peptide forming reaction led to the use of terminally modified tRNA for studying the mechanism of peptide bond synthesis. Yeast Phe-tRNA^{ox-red} was used, in which the $C_{2'}$, $C_{3'}$ bond in the terminal 3'-adenosine was cleaved by periodate oxidation and subsequent borohydride reduction. The Phe-tRNA^{ox-red} and the 3'-terminal fragments derived from it, namely CACCA^{ox-red}(Phe) and Ado^{ox-red}(Phe) were used in the experiments to be described below.

a. Characteristics and properties of oxidized-reduced tRNA

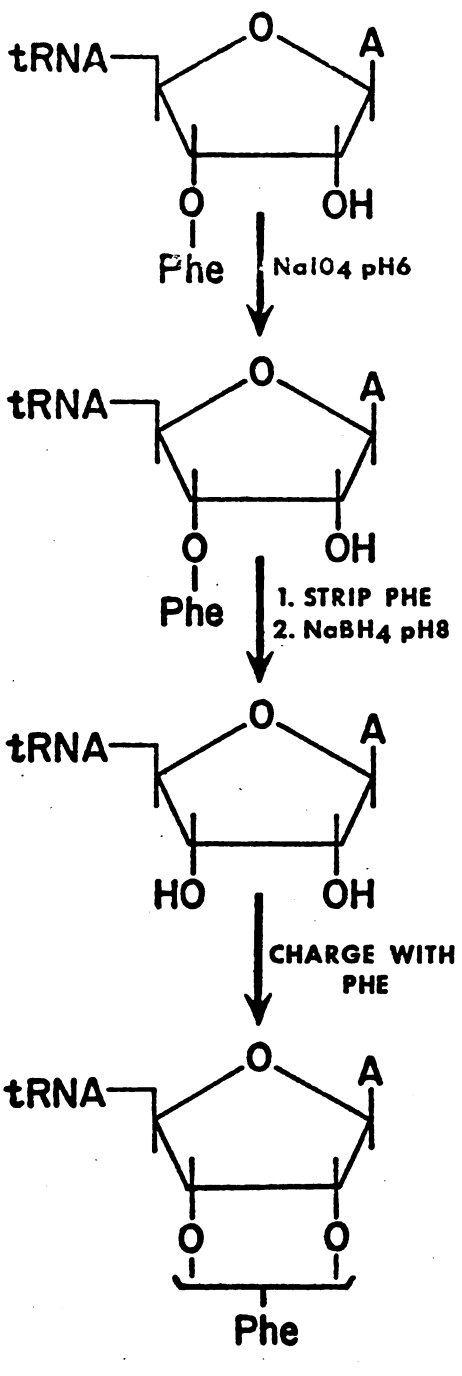
The scheme for preparation of the oxidized-reduced tRNA^{Phe} is presented in Figure 9. In the control tRNA, the 2', 3'-hydroxyl group was first blocked by enzymatic aminoacylation with phenylalanine and

Figure 9

Scheme for the preparation of
Phe-tRNA^{ox-red} and Phe-tRNA^{cont}



PHE-tRNA OX-RED



PHE-tRNA CONTROL

then oxidized with periodate. Under these conditions, no reaction took place since periodate can only oxidize vicinal hydroxyl groups. In the second step, phenylalanine was deacylated (using conditions which are known not to alter the biological properties of tRNA) before $NaBH_4$ reduction so that the $tRNA^{cont}$ would be as much like the oxidized tRNA as possible. Any other oxidative or reductive reaction occurring elsewhere in the tRNA molecule should be equally present in the Phe-$tRNA^{cont}$ as well as in the $tRNA^{Phe}$. As reported earlier (Chen and Ofengand, 1970; Cramer et al., 1968) oxidized-reduced $tRNA^{Phe}$ can be acylated by its homolagous Phe-tRNA synthetase. Since the charging ability of oxidized tRNA was zero, complete oxidation must have occurred. This is also documented by the failure to detect any Ado(Phe) in Phe-$tRNA^{ox-red}$ (see Figure 5). Inactivation was not due to oxidative inactivation elsewhere in the molecule, since oxidation of Phe-tRNA did not affect its subsequent acceptance activity. Reduction to the diol must have proceeded to at least 83-96% of completion since acceptance was regenerated to this level.

The enzymatic acylation of tRNA lacking the carbon-carbon bond between the 2' and 3' positions is neither unique to the yeast Phe-tRNA synthetase system nor is it universal. Unfractionated *E. coli* $tRNA^{ox-red}$ could accept met, phe, and tyr (Ofengand and Chen, 1972; Tal et al., 1972) but not arg, tryp, hist, ileu, val, asp or ser (Tal et al., 1972; Mehler, 1970). This suggests that although the terminal adenosine is universally present in all tRNA species, the intactness of the covalent bond between the C'_2 and C'_3 of the ribose moiety may be a decisive determinant for the aminoacylation of some tRNA species, but plays a minor role with others.

As for the biological properties of yeast Phe-tRNA^{ox-red}, Chen and Ofengand (1970) reported that it could no longer form a ternary complex with E. coli Tu factor and GTP. The modification also blocked T-factor dependent Phe-tRNA binding to the A site on the ribosome and partially inhibited non-enzymatic binding at the P site. Furthermore, Phe-tRNA^{ox-red} was unable to make polyphenylalanine in a tRNA-dependent cell-free polypeptide-synthesizing system.

b. Binding studies with oxidized-reduced tRNA^{phe}

According to the generally believed concept of protein biosynthesis, peptide bond formation on ribosomes involves at least two binding sites for tRNA (Lucas-Lenard and Lipmann, 1971; Monro et al., 1968). One, for holding the peptidyl-tRNA (P-site) and the other for attachment of the aminoacyl-tRNA (A-site). During various stages of protein synthesis, peptidyl-tRNA is bound to the ribosomes at either of the binding sites. Before a peptide bond is formed, the peptidyl tRNA (or initiator fMet-tRNA or N-acetyl-Phe-tRNA) remains bound to the P-site but immediately after peptide bond formation, the peptidyl moiety is bound to the tRNA at the A-site. The newly formed peptidyl-tRNA is then moved from the A-site to the P-site by the action of translocase (and GTP) and the evacuated A-site is occupied by another molecule of incoming aminoacyl-tRNA and the process is repeated. The puromycin reaction has been widely used for the identification of the binding sites (Monro et al., 1968; Tanaka et al., 1972; Yarmolinsky and de la Haba, 1959). Peptidyl-tRNA bound to the P-site reacts directly with puromycin (as an analogue of AA-tRNA, hence A-site substrate) without the addition of translocase,

while peptidyl-tRNA bound to the A-site reacts with puromycin only after a translocation catalyzed by G-factor and GTP occurs (Traut and Monro, 1964). N-acetyl-Phe-tRNA like fMet-tRNA binds to the P-site on ribosomes (Lucas-Lenard and Lipmann, 1967) and forms N-acetyl-Phe-puromycin in the presence of added puromycin. This reaction, catalyzed by ribosomal peptidyl transferase, has been widely used as a model for peptide bond formation (Lucas-Lenard and Lipmann, 1971).

Since an important prerequisite for the puromycin reaction is the correct binding of the P-site substrate to the ribosome, binding of N-acetyl-Phe-$tRNA^{ox-red}$ under the conditions of the puromycin reaction 20 mM Mg^{++}) was examined (Table V). The binding of Phe-$tRNA^{ox-red}$ was previously reported by Ofengand and Chen (1972) as a function of Mg^{++} concentration. It is clear that the binding of untreated N-acetyl-Phe-tRNA and N-acetyl-Phe-$tRNA^{cont}$ took place quantitatively and that about 90% of the input N-acetyl-phe-$tRNA^{ox-red}$ was bound at all concentrations tested. Since it has been reported that at this high magnesium concentration (20 mM), all the N-acetyl-Phe-tRNA bound to ribosomes in the presence of poly U is not located on the P-site (Weissbach et al., 1968), it was important to explore the precise location of the binding. This was done in two ways. First, in order to prevent binding at the A-site, the experiment was repeated in the presence of tetracycline, an antibiotic which specifically blocks binding to this site (Sarkar and Thach, 1968). At 0.4 mM tetracycline, the same amount of both control and oxidized-reduced N-acetyl-Phe-tRNA were bound (85% oxidized-reduced, 92% control), suggesting that almost all of the binding took place at the P-site. The results shown in the table represent the maximum extent of binding since preliminary experiments showed that maximum binding occurred in 5 minutes

TABLE V

Binding of N-acetyl-Phe-tRNA^{ox-red} to ribosomal P site

N-Acetyl-Phe-tRNA Added (pmoles)	N-Acetyl-Phe-tRNA Bound (pmoles)		
	untreated	control	ox-red
4	4.1 (4.0)	4.0 (4.0)	3.6 (3.6)
8	8.0 (8.0)	7.9 (8.0)	6.7 (7.6)
12	12.4 (12.1)	11.3 (12.1)	8.8 (10.7)
16	15.2 (16.2)	13.3 (16.0)	12.4 (14.3)
20	19.6 (20.5)	17.3 (20.0)	15.7 (16.4)

Binding of N-acetyl-Phe-tRNA to ribosomes was measured by the procedure described by Nirenberg and Leder (1964).

The reaction mixture in 0.1 ml contained 0.05 M Tris-acetate, pH 7.4, 0.1 M NH_4Cl, 0.08 M KCl, 0.02 M $MgCl_2$, 10 µg Poly U, 0.01 M DTT, 4 A_{260} units of activated ribosomes and different amounts of N-acetyl-Phe-tRNA (control, untreated or oxidized-reduced) as indicated. Incubations were at 35° C for 10 minutes and reactions were terminated by the addition of 2 ml of a cold wash buffer containing 0.05 M Tris-acetate, pH 7.4, 0.1 M NH_4Cl, 0.02 M $MgCl_2$, and 0.08 M KCl, and the mixture filtered through a pre-washed Millipore filter. The filter was washed three times with 2 ml of cold wash buffer, dissolved in 10 ml of Bray's solution and counted. In experiments with tetracycline, the reaction mixture was pre-incubated with 0.4 mM tetracycline for 5 minutes at 35° C. At the end of it the substrate was added and the procedure followed as described above. Values in parentheses were obtained in absence of tetracycline.

at 20 mM magnesium and that ribosomes were in excess with or without tetracycline. The presence of excess ribosomes at all N-acetyl-Phe-tRNA concentrations tested is also implicit in the proportionality of the N-acetyl-Phe-tRNA bound to the N-acetyl-Phe-tRNA added that is observed in the experiment. Second, it was shown by the addition of puromycin and 0.5 mM Fusidic acid, a translocase inhibitor (Tanaka et al., 1968) (data not shown) that 92% of the bound N-acetyl-Phe-$tRNA^{cont}$ was puromycin-reactive and hence at the P-site. N-acetyl-Phe-$tRNA^{ox-red}$ could not be tested in this way because it does not form a puromycin product (see section 2.a.). This result indicates that the amount of N-acetyl-Phe-puromycin formed did not involve any translocation during peptide bond formation and that all of the bound N-acetyl-Phe-$tRNA^{ox-red}$ was at the P-site on the ribosome.

These binding results are in apparent contradiction with some earlier reports (Suzuka et al., 1970; Tanaka et al., 1972; Ishitsuka and Kaji, 1972) that at 13-30 mM magnesium concentrations, N-acetyl-Phe-tRNA binds to both A and P-sites. In view of the differential inhibitory effects of unacylated tRNA on the binding of N-acetyl-Phe-tRNA, interpretation of binding experiments is not always easy. It is known that non-enzymic binding of deacylated tRNA occurs preferentially at the P-site (Seeds et al., 1967; Ofengand and Henes, 1969) and it therefore, interferes with the binding of AA-tRNA and peptidyl-tRNA. It should be noted that pure preparations were used in this study and N-acetyl-Phe-tRNA was never in excess. The present results, however, are consistent with those of deGroot and Lapidot (1971). Using pure preparations of $(Gly)_3$-Phe-$tRNA^{Phe}$, they observed that tetracycline did not inhibit the

binding of peptidyl-$tRNA^{Phe}$ at 30 mM $Mg(OAc)_2$ indicating that binding to the P-site was direct. On the other hand, peptidyl-tRNA from crude preparations bound more to the A-site than to the P-site at 30 mM $Mg(OAc)_2$ under the same experimental conditions (DeGroot and Lapidot, 1971).

c. Bromoacetyl-Phe-$tRNA^{ox-red}$ binding to ribosomes and subunits

Experiments reported in Section 2.c. and 2.d. were performed in collaboration with Maria Pellegrini of Dr. Charles Cantor's laboratory at Columbia University.

As an extension of the binding studies reported in Table V, α-bromoacetyl-Phe-$tRNA^{ox-red}$, an analogue of peptidyl-tRNA which reacts with free amino groups in proteins, i.e., lysine residues, was used as an affinity label to define more precisely the nature of N-Ac-Phe-$tRNA^{ox-red}$ binding to the ribosome. Recently, using α-bromoacetyl-Phe-tRNA as the affinity label, Cantor and his co-workers (Pellegrini et al., 1972) identified the 50S proteins (L_2 and L_{27}) to which the label attached covalently in a poly U-dependent binding to 70S ribosomes. It is known that peptide bond forming activity is localized exclusively in the 50S ribosomal particle (Monro, 1967; Maden, 1968). In the present studies, specifically, the question asked was whether N-Ac-Phe-$tRNA^{ox-red}$ was positioned at the P site such that it could label the same proteins as the control, despite the modification at the 3' end. Table VI shows the extent of poly U dependent binding of control and oxidized-reduced BrAc-Phe-tRNAs compared to the binding of their respective N-acetyl-Phe-tRNAs. The binding of BrAc-Phe-$tRNA^{cont}$ to 70S ribosomes confirms the earlier report (Pellegrini et al., 1972) which used untreated BrAc-Phe-tRNA, and BrAc-Phe-$tRNA^{ox-red}$ showed similar binding. Ribosomes from each sample listed in the table were separated into 50S and 30S particles by dialysis and subsequently analyzed on sucrose gradients

TABLE VI

Comparison of Binding of Affinity Analogs

of Peptidyl-tRNA^{ox-red} and Peptidyl-tRNA^{cont}

Peptidyl tRNA Added[a]	cpm[^{3}H]Phe bound/nmole		
		Dissociated 70S[c]	
	70S[b]	50S	30S
N-Ac[^{3}H]Phe-tRNA^{cont}	17100	110	0
N-Ac[^{3}H]Phe-tRNA^{ox-red}	26500	310	46
Br-Ac[^{3}H]Phe-tRNA^{cont}	18300	620	280
Br-Ac[^{3}H]Phe-tRNA^{ox-red}	27300	1380	160

[a] The incubation mixture for binding contained 400 A_{260} units of 70S ribosomes, 20 A_{260} units of Poly U, 4.2 pmoles (93,000 cpm) of BrAc[^{3}H]Phe-tRNA^{cont}, 5.4 pmoles (120,000 cpm) of BrAc[^{3}H]Phe-tRNA^{ox-red}, 5 pmoles (110,000 cpm) each of N-Ac[^{3}H]Phe-tRNA^{cont} or N-Ac[^{3}H]Phe-tRNA^{ox-red} in 0.75 ml of buffer A (10 mM Mg^{++}) as described in Chapter II.C.3.

[b] The mixture was incubated at 37° C. After 30 minutes 0.2 ml of the mixture was layered over a 5-25% sucrose gradient in buffer A (5.2 ml) and centrifuged in an SW 50.1 rotor at 40,000 rpm for 1.2 hours. Fractions were collected, the ribosome pattern was obtained by UV-absorption measurement and the bound [^{3}H]Phe was determined as TCA precipitable cpm.

[c] 0.2 ml of the incubated mixture was dialyzed in buffer B (0.2 mM Mg^{++}) described in Chapter II,C.3., to dissociate ribosomes into 50S and 30S subunits. It was then layered over a 10-30% sucrose gradient in buffer B (5.2 ml) and centrifuged as above for 2 1/4 hours. Fractions were analyzed as described above.

(Table VI., Figure 10). The samples containing N-acetyl-phenylalanyl derivatives of both control and oxidized-reduced tRNA lost practically all of their bound tRNA by this treatment while both BrAc-Phe-tRNA^{cont} and BrAc-Phe-tRNA^{ox-red} remained associated with both subunits, although primarily with the 50S subunit. It seems clear that BrAc-Phe-tRNA^{ox-red} becomes covalently linked to the 50S subunit just as does BrAc-Phe-tRNA^{cont}. These results are in agreement with the results of Cantor and his co-workers (1972) who showed that untreated BrAc-Phe-tRNA attaches co-valently at the P-site.

d. Br-Ac-Phe-tRNA^{ox-red} attachment to 50S proteins

Results presented thus far suggest that BrAc-Phe-tRNA^{ox-red} like BrAc-Phe-tRNA^{cont} binds to the P-site and becomes covalently attached to ribosomes. To provide more convincing evidence and to narrow down the possible sites of attachment, 50S particles containing BrAc-Phe derivatives of control and ox-red tRNAs were separated into RNA and a total 50S protein fraction by treatment with LiCl-urea (Traub and Nomura, 1968). Subsequently, the protein fraction was analyzed by poly-acrylamide gel electrophoresis. Table VII shows the data from the analysis of a 2-D gel separation of 50S proteins from BrAc-Phe-tRNA reacted ribosomes. The protein which is radioactively labelled to the greatest extent is L_2 in both control and oxidized-reduced samples; the latter also binds appreciably to L_{27}. It was indicated (Dr. C. Cantor, personal communication) that under these conditions, BrAc-Phe-tRNA which contained less unaminoacylated-tRNA^{Phe} showed less labeling of L_{27} and a higher relative labeling of L_2. Therefore, binding of the oxidized-reduced

Figure 10

Retention of Bromoacetyl-Phe-tRNA^{ox-red} on

50S and 30S Ribosomes

Experimental details are described in Table VI. under a. and c.

 , A_{260};  , cpm [^{3}H]Phe. (a) N-acetyl[^{3}H]Phe-tRNA^{ox-red} (b) N-acetyl[^{3}H]Phe-tRNA^{cont} (c) Bromoacetyl[^{3}H]Phe-tRNA^{ox-red} and (d) Bromoacetyl[^{3}H]Phe-tRNA^{cont}.

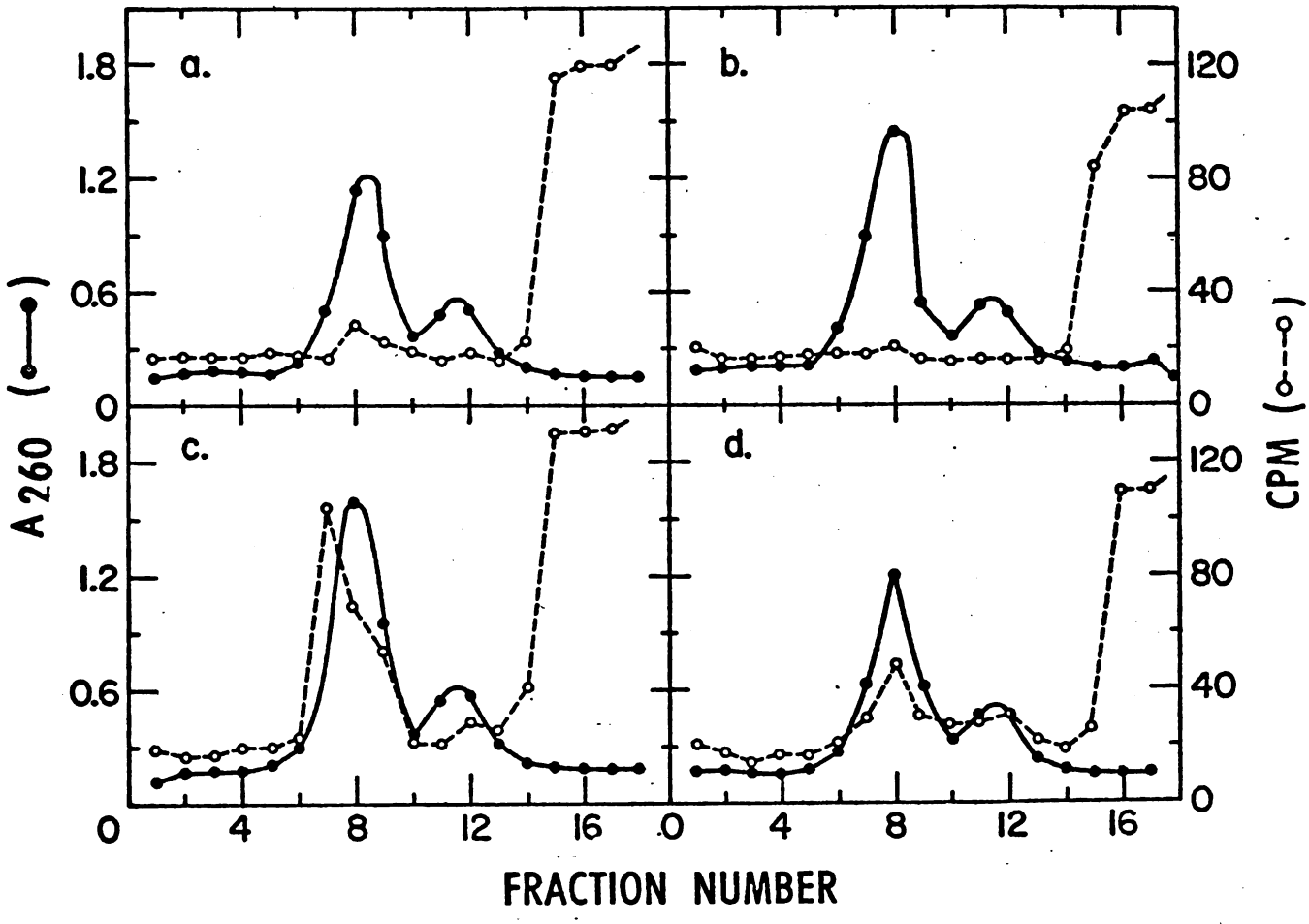

a.
b.
c.
d.
A260 (●—●)
CPM (○—○)
1.8
1.2
0.6
0
120
80
40
0
4
8
12
16
FRACTION NUMBER

TABLE VII

Covalent Attachment of BrAc-Phe-tRNA^{ox-red} to

50S Ribosomal Proteins

50S Proteins	BrAc[^{3}H]Phe-tRNA^{cont} (cpm)	BrAc[^{3}H]Phe-tRNA^{cont} + excess tRNA (cpm)	BrAc[^{3}H]Phe-tRNA^{ox-red} (cpm)
L 2	1680	186	2894
L 3	0	0	412
L 14	376	53	0
L 15	177	46	77
L 16	65	77	252
L 27	0	80	1185

50S particles were isolated from BrAc[^{3}H]Phe-tRNA^{ox-red} and BrAc[^{3}H]Phe-tRNA^{cont} reacted 70S ribosomes as described in Table Proteins were separated from RNA by the LiCl-urea method (Traub and Nomura, 1968) and treated with RNase at 25° for 30 minutes to digest away any remaining rRNA and tRNA. The proteins were then analyzed by two dimensional polyacrylamide gel electrophoresis according to the method of Kaltschmidt and Wittmann (1970). Gels were stained in 0.01% Coomassie Brilliant Blue in 10% TCA (Crambach et al., 1967). Each protein spot was cut out and TCA was removed from these gel pieces by ether extraction. They were then oxidized in a Packard Tritium Oxidizer and the radioactivity counted. Identification of individual proteins and their nomenclature was according to Kaltschmidt and Wittman (1970).

sample to L_{27} might be attributed to the differential relative proportion of deacylated tRNA species in the samples used (see chapter II.B. 9.). Alternatively, the difference may reflect the degree of structural dissimilarity between the terminus of the two tRNA species. As can be seen in the table, a few of the other 50S proteins (L_{14}, L_{15} in control, L_3, L_{16} in ox-red) also seem to be radioactively labeled, though to a much lesser extent than L_2 and L_{27}. Clear separation of individual proteins in these regions have not been very successful so far. Therefore, overlap of some of these protein spots on the gels causes ambiguity about which proteins are actually labelled (Pellegrini et al., 1972). In any case, they are quantitatively not very important.

In order to be sure that the labeling observed was not due to a reaction of free Bromoacetylphenylalanine liberated by hydrolysis of the derivatized-tRNA during the course of the processing steps, a control experiment was performed in which Br-Ac[^{3}H]Phe-tRNA was allowed to react with ribosomes in the presence of excess unacylated tRNA in the standard incubation mixture. As shown in the table, the amount of radioactivity associated with L_2 in the control experiment was reduced to 7-11% of the samples containing Br-Ac-Phe-$tRNA^{cont}$ or Br-Ac-Phe-$tRNA^{ox-red}$.

Since the results using $tRNA^{ox-red}$ were positive, it was necessary to be sure that the radioactivity associated with protein was actually derived from Br-Ac-Phe-$tRNA^{ox-red}$ and not from contaminating Br-Ac-Phe-$tRNA^{control}$. For example, it was considered possible that the longer incubation required for charging of $tRNA^{ox-red}$ with synthetases may have removed most of the terminal $adenosine^{ox-red}$ (due to RNase contamination) which in turn was replaced by adenosine (catalyzed by nucleotidyl transferase) and was then charged with phenylalanine. However, the

analysis described in Chapter II (Figure 7) rules out the above hypothesis. Analysis of the pancreatic RNase digestion products from both control and oxidized-reduced Br-Ac-Phe-tRNAs by TLC confirms that the products are Br-Ac-Phe-adenosine and Br-Ac-Phe-adenosine^{ox-red} respectively and that there is no (< 0.8%) Br-Ac-Phe-adenosine in the oxidized-reduced sample. This point is important also in view of the low yield of covalently bound Br-Ac-Phe-tRNA. In this experiment, 0.22% and 0.51%, respectively, of the control and oxidized-reduced species added to the first incubation covalently bound to 50S subunits. However, since there was < 0.8% of control tRNA in the ox-red preparation, it could not account for the results observed.

Although the Br-Ac-Phe-tRNA moiety or moieties in L_2 and L_{27} proteins have not been identified, convincing data exist (Oen et al., 1973) which argue that L_2 is located near to the P-site while L_{27} may be in the region of the A-site (Dr. C. Cantor, personal communication). Evidence for the positioning of L_2 at the P-site is the fact that simultaneous binding of cold Br-Ac-Phe-tRNA and radioactive [^{3}H]Phe-tRNA^{phe} leads to covalent attachment of the radioactive amino acid to L_2 predominantly. In order for this to occur, both tRNA moieties must be in the correct position for peptidyl transfer. A similar result has been obtained by the reaction of radioactive puromycin with non-radioactive Br-Ac-Phe-tRNA bound to the ribosome.

In consideration of the above, it appears that Br-Ac-Phe-tRNA^{ox-red} binds at the peptidyl site of the 50S subunit where it reacts primarily with protein L_2 and at least by this criteria is structurally equivalent to normal tRNA.

e. Formation of initiation complex with N-acetyl-Phe-tRNA^{ox-red}

The initiation of protein synthesis in E. coli proceeds with the formation of a complex consisting of mRNA, a 70S ribosome, and a specific initiator fMet-tRNA. However, N-acetyl-Phe-tRNA has been shown to mimic fMet-tRNA$_f^{Met}$ as an initiator of protein synthesis in vitro (Lucas-Lenard and Lipmann, 1971). Formation of such complexes requires GTP, Mg^{++} and NH_4^+ and in addition, several proteins called initiation factors that are loosely associated with ribosomes and can be dissociated by high salt treatment (Haselkorn and Rothman-Denes, 1973). Three distinct protein factors (IF-1, IF-2 and IF-3) are involved in the initiation step. According to current concepts (Haselkorn and Rothman-Denes, 1973) the first step in the formation of the initiation complex is the association of IF-3 with the 30S subunit and mRNA (Sobol et al., 1973; Benne et al., 1973). The resulting complex is next joined by another complex consisting of IF-2, GTP and the initiator tRNA (Benne et al., 1973; Lockwood et al., 1971). To this IF-1 binds and then IF-3 is dissociated (Lelong et al., 1970). Subsequent addition of the 50S subunit forms the 70S initiator-tRNA-mRNA initiator complex with the concomitant release of IF-1, IF-2 and GDP.

It was previously demonstrated (Ofengand and Chen, 1972) that enzymatic binding of Phe-tRNA^{ox-red} to the A site on the ribosome was completely blocked. It was therefore of interest to see whether the modified tRNA could participate in a similar binding reaction, namely initiation complex formation with the 30S subunit. The formation of such a complex in the presence of E. coli crude initiation factors was studied using N-acetyl-Phe-tRNA^{ox-red} (Table VIII). It is clear that N-Ac-Phe-tRNA^{cont} forms the initiation complex efficiently at 4 mM Mg^{++} while

TABLE VIII

Enzymatic Binding of N-Acetyl-Phe-tRNA^{ox-red} and N-Acetyl-Phe-tRNA^{control} to 30S Subunits

System	N-acetyl-Phe-tRNA Bound	
	Control	Oxidized-reduced
	pmoles	pmoles
Complete	6.32	0.05
-GTP	1.32	0.01
-initiation factors	1.28	0.02
-poly U	0.26	0.04

The determination of initiation factor dependent binding to 30S subunits was based on the Millipore filtration procedure of Nirenberg and Leder (1964) which measures N-acetyl-phe-tRNA ribosome complexes bound to nitrocellulose filters.

Each assay mixture (0.05 ml) contained 50 mM Tris-acetate pH 7.4, 50 mM potassium acetate, 4 mM $MgCl_2$, 1 mM GTP, 3.6 A_{260} units of 30S ribosomal subunits, 208 µg of crude initiation factor, 10 µg Poly U and 20 pmoles each of N-acetyl-phe-tRNA. After incubation at 22° for 8 minutes, the mixture was diluted with 1 ml of a cold washing buffer containing 50 mM Tris-acetate pH 7.4, 160 mM NH_4Cl and 12 mM $MgCl_2$ and passed through a Millipore filter (HAWP 0.45µ). After 3 washes with 2 ml of cold buffer, the filter was dissolved in 10 ml of Bray's (Bray, G.A. 1960) solution and counted.

N-Ac-Phe-tRNA^{ox-red} is unable to form such a complex under the same conditions. Furthermore, binding is dependent on the presence of initiation factors, poly U and GTP. The effect of N-Ac-Phe-tRNA concentration and the kinetics of the reaction are shown in Figure 11. With N-Ac-Phe-tRNA^{cont}, maximal binding was obtained (30% of input) when the input concentration of the N-Ac-Phe-tRNA^{cont} was 18 pmoles (Figure 11B). Using this concentration, as seen in Figure 11A, the binding to 30S ribosomes was complete in 4 minutes. Parallel experiments using N-Ac-Phe-tRNA^{ox-red} showed no detectable complex formation. The experiments in Table VIII and Figure 11 support the view that N-Ac-Phe-tRNA^{ox-red} is not recognized by the E. coli initiation factors and thereby it is unfit for initiation factor-dependent binding to 30S ribosomes. In this respect, the results parallel those found for EFT-dependent binding to the A site.

Figure 11

Initiation factor dependent binding of N-acetyl-Phe-tRNA^{ox-red} to 30S subunits.

A. Time course. The reaction mixture and assay was as described in Table VIII using 18 and 20 pmoles of control and oxidized-reduced N-acetyl-Phe-tRNA, respectively. Incubation was at 22° C for the indicated time. The amount of radioactivity retained on the filter in the absence of 30S subunits was subtracted from each value.

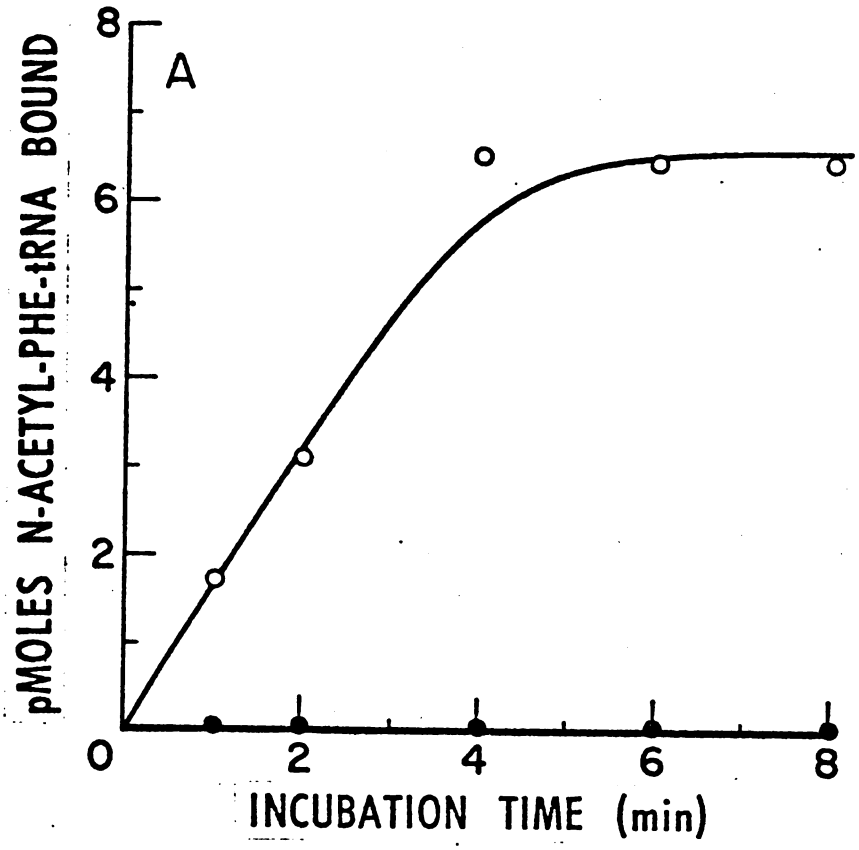
○ , control

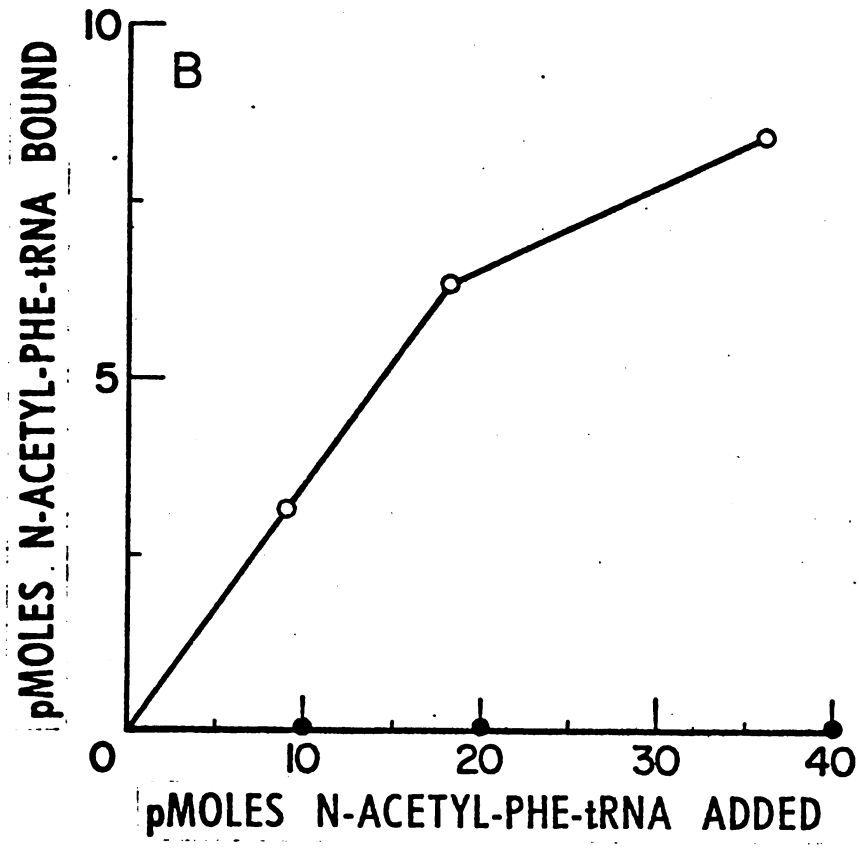
● , oxidized-reduced.

B. N-acetyl-Phe-tRNA concentration dependence. Components of the binding mixture were as in A except that the amount of tRNA was varied as indicated. Incubation was at 22° for 8 minutes.

○ , control

● , oxidized-reduced.


A
pMOLES N-ACETYL-PHE-tRNA BOUND
0
2
4
6
8
INCUBATION TIME (min)


B
10
5
0
10
20
30
40
pMOLES N-ACETYL-PHE-tRNA BOUND
pMOLES N-ACETYL-PHE-tRNA ADDED

2. Peptide bond formation with puromycin

The puromycin reaction is considered to be a prototype of the reaction by which peptide bonds are formed during protein biosynthesis (Allen and Zamecnik, 1962; Nathans, 1964; Smith et al., 1965) since the antibiotic is an analog of the adenosine aminoacyl terminus of aminoacyl-tRNA (Yarmolinsky and de la Haba, 1959). When added to a ribosomal system during polypeptide synthesis, it is linked by a peptide bond to the carboxyl end of the growing polypeptide, which then become detached from the ribosome, no longer being bound to it by a molecule of tRNA. It is known that unlike aminoacyl-tRNA, puromycin-mediated peptide bond formation does not require supernatant factors, mRNA, GTP or even 30S ribosomal subunits (Traut and Monro, 1966; Maden et al., 1966). Consequently, the puromycin reaction has been commonly used for the study of peptidyl transferase-catalyzed peptide bond formation with P-site substrates, e.g., N-Ac-Phe-tRNA (Haenni and Chapeville, 1966), fMet-tRNA (Zamir et al., 1966), Polylysyl-tRNA (Rychlik, 1966), Poly-Phe-tRNA (Traut and Monro, 1964), and peptidyl-tRNA (Allen and Zamecnik, 1962).

a. Donor-site activity of N-acetyl-Phe-tRNA^{ox-red} and CACCA^{ox-red}(Phe) on 70S and 50S ribosomes

To test whether the interaction of N-Ac-Phe-tRNA^{ox-red} with ribosomes leads to formation of the peptide bond, the puromycin reaction was utilized. N-Ac[^{14}C]Phe-tRNA^{ox-red} made by acetylation of [^{14}C]Phe-tRNA^{ox-red} with acetic anhydride (99% acetylated) (Haenni and Chapeville, 1966) was reacted with puromycin the presence of activated ribosomes

(Miskin et al., 1970), and ethyl acetate extractable radioactivity was measured. It is known that N-Ac-Phe-puromycin can be extracted from a pH 5.5 aqueous phase and that extraction is specific for uncharged derivatives of labelled amino acids in which both the amino and carboxyl groups are blocked (Leder and Bursztyn, 1966; Maden and Monro, 1968). Figure 12 shows the time course of N-Ac-Phe-puromycin formation for the oxidized-reduced and its equivalent control N-Ac-Phe-tRNA. An untreated(N-Ac-Phe-tRNA^{unt}) was also included as a reference. As seen in the figure, peptide bond formation with control and untreated samples reached a plateau at 20 minutes by which time 67% and 95% of the respective donors were converted to N-Ac-Phe-puromycin. Under the same conditions, there was no detectable peptide formed with the oxidized-reduced sample although only 2% ester hydrolysis took place during the course of the incubation. In a separate experiment, puromycin concentrations as high as 20 mM and 40 minute incubation were also used but with no effect. Since peptidyl transferase-mediated peptide bond formation can take place with 50S subunit alone, and since oxidized-reduced tRNA binds to 50S proteins (Section 1.d.) the puromycin reaction was tried with this subunit in the presence of methanol as described by Miskin et al., (1970). In this assay also N-Ac-Phe-tRNA^{ox-red} was unable to form a puromycin product whereas the control and the untreated donors were equally active (Figure 13). At a concentration of 1.0 mM puromycin, 14% of the control N-Ac-Phe tRNAs formed a peptide product in 30 minutes.

In order to test the effect of oxidation-reduction on tRNA more rigorously, CACCA(Ac-Phe) and CACCA^{ox-red}(Ac-Phe), obtained by hydrolysis of their respective Ac-Phe-tRNAs, were used in the "fragment reaction" described by Monro and Marcker (1967). This assay is thought to be the

Figure 12

N-acetyl-Phe-puromycin formation from N-acetyl-Phe-tRNA^{ox-red} and puromycin using 70S ribosomes as a function of time

The assay is described in Chapter II.D.1.a. Donor substrates in the amounts shown below were added in a total volume of 0.1 ml. Puromycin was added last and the mixture incubated at 35° C for the times indicated. Reactions were terminated and N-acetyl-Phe-puromycin extracted and counted as described. The results are presented as percent of N-Ac-phe-tRNA added.

○ , 15 pmoles N-acetyl[^{14}C]Phe-tRNA^{cont}

● , 22 pmoles N-acetyl[^{14}C]Phe-tRNA^{unt}

■ , 24 pmoles N-acetyl[^{14}C]Phe-tRNA^{ox-red}

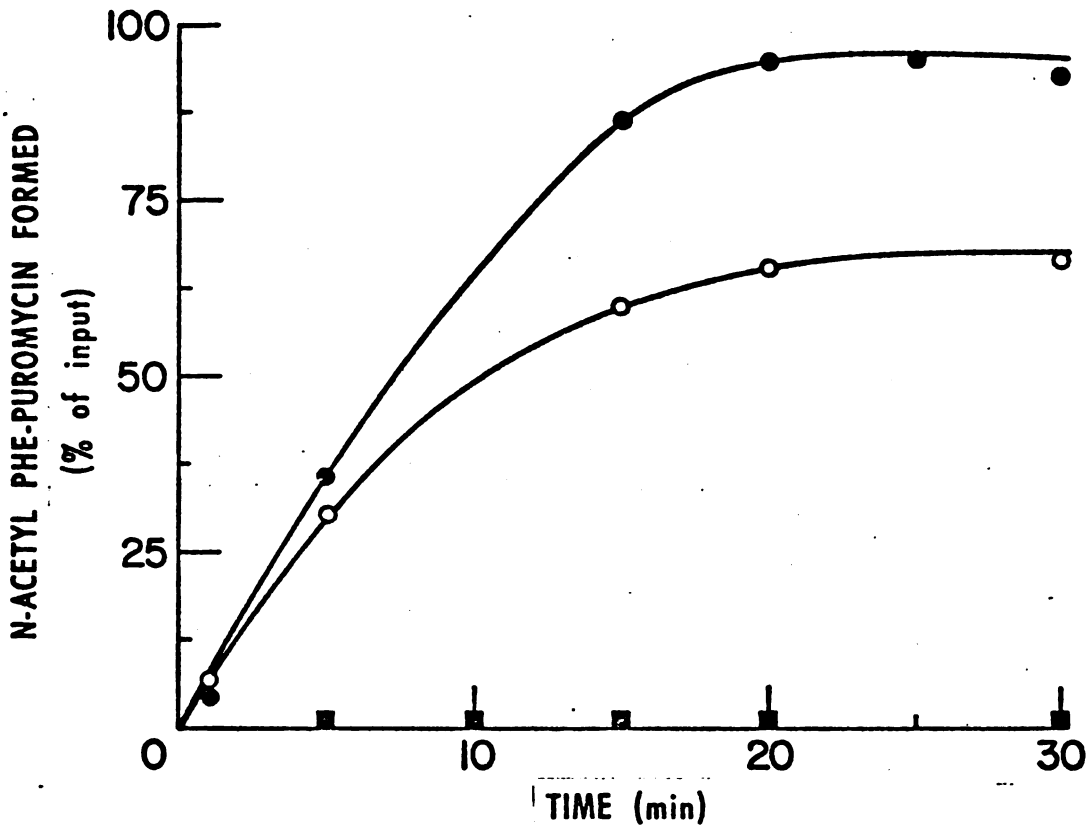

100
75
50
25
0
10
20
30
N-ACETYL PHE-PUROMYCIN FORMED
(% of input)
TIME (min)

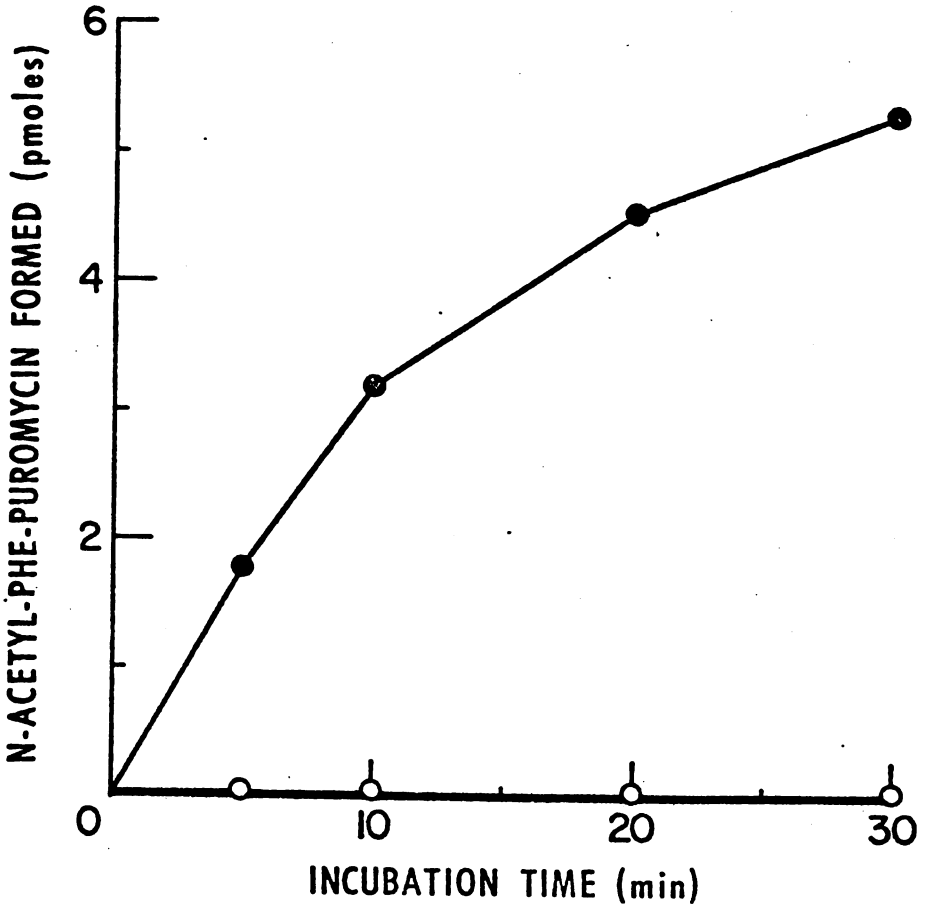
Figure 13

Time course of N-acetyl-Phe-puromycin synthesis on 50S ribosomes

Assays were performed as described in Chapter II.D.1.b. The donor substrates were added last and the reaction started by adding 25 µl of methanol. After incubation the samples were processed as described. 5.4 A_{260} units of 50S subunits were used. Incubation was at 22° C for the indicated times in the presence of 1 mM puromycin.

● , 38 pmoles of N-acetyl[^{14}C]phe-tRNA^{cont}

○ , 48 pmoles of N-acetyl[^{14}C]phe-tRNA^{ox-red}


6
4
2
0
10
20
30
N-ACETYL-PHE-PUROMYCIN FORMED (pmoles)
INCUBATION TIME (min)

direct way of investigating the catalytic center of peptidyl transferase itself, uncomplicated by any side effects of a necessity for tRNA to bind to the ribosome (Monro and Marcker, 1967; Monro, 1971). Figure 14 shows that in confirmation of the above results, oxidation-reduction completely inactivated the tRNA fragment for reaction as a peptidyl donor using puromycin as an acceptor. Since only 5 nucleotides are involved, the effect cannot be due to any alteration at some distant point in the molecule, nor is it likely to be due to some distortion of tertiary structure due to opening of the terminal ribose ring. It should be emphasized that the control fragment (described in Chapter II.B.7.) was exposed to the same chemical procedures. Finally, there can be no question that acetylation of the $tRNA^{ox-red}$ produced any artifact since the data in Chapter II.B.6.c. shows clearly that acetylation took place only on the amino group of the amino acid.

b. Acceptor Site activity of Phe-$tRNA^{ox-red}$ and $CACCA^{ox-red}$(Phe) on 70S and 50S Ribosomes

Since it is known that the donor and acceptor sites on ribosomes possess differential specificity (Rychlik et al., 1970), the following experiments were done to test whether Phe-$tRNA^{ox-red}$ was functional in peptide bond formation from the A-site.

Using N-Ac-Phe-tRNA as donor in a ribosome-poly U system, the ability of Phe-$tRNA^{ox-red}$ to act as an acceptor in the formation of N-Ac-Phe-Phe was tested in the assay system described by Lucas-Lenard and Haenni (1968). Binding of the donor substrate was ensured before addition of the acceptor. Consequent peptide bond formation was determined by measurement of the

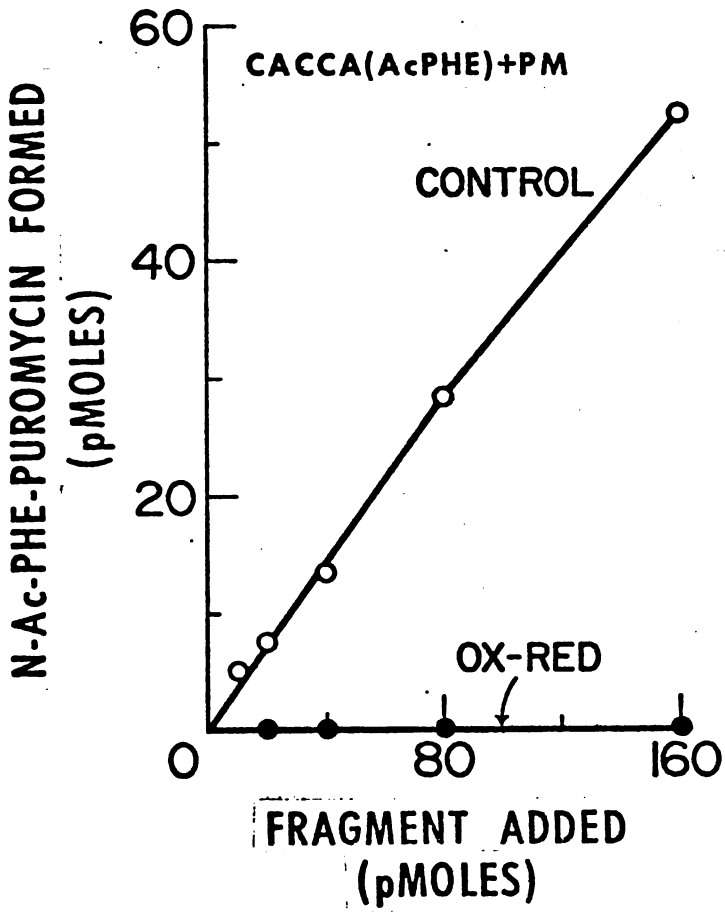
Figure 14

Formation of N-acetyl-Phe-puromycin from oxidized-reduced N-acetyl-Phe-pentanucleotide and puromycin

Components of the assay mixture were as described in Chapter II.D.1.b with activated 70S ribosomes (Miskin et al. 1970) and a final volume at 0.05 ml. N-acetyl[^{14}C]Phe-pentanucleotide fragments were added last, the reaction initiated by adding 25 µl of methanol, and incubated for 40 minutes at 30° C. Reactions were terminated by adding 5 µl of 10 N NaOH and incubating for 5 minutes at 37° C (Miskin et al. 1970). 0.5 ml of 1 M potassium phosphate pH 7.0, was then added and the reaction mixture extracted with ethylacetate and counted as described in Chapter II.D.1.b.

○ , $CACCA^{cont}$ (Ac-[^{14}C]Phe)

● , $CACCA^{ox-red}$ (Ac-[^{14}C]Phe)


60
40
20
0
80
160
CACCA(AcPHE)+PM
CONTROL
OX-RED
N-Ac-PHE-PUROMYCIN FORMED
(pMOLES)
FRAGMENT ADDED
(pMOLES)

amount of N-Ac-Phe-Phe formed. The result of such an experiment is shown in Table IX and Figure 15. It is clear from the table that although Phe-tRNA^{ox-red} was bound to the A site of the ribosomes as efficiently as Phe-tRNA^{cont} (45% and 54% respectively, of their input), it failed to show comparable ability to participate in peptide bond formation. Less than 4% of the bound Phe-tRNA^{ox-red} (14.5 pmoles bound) accepted the peptide moiety from N-Ac-Phe-tRNA whereas 34% of the bound Phe-tRNA^{cont} participated in this reaction. In other words, Phe-tRNA^{ox-red} is less than 12% as active as Phe-tRNA^{cont} as an acceptor. Note that the product, N-Ac-Phe-Phe, contained equal proportions of ^{14}C and ^{3}H ($^{14}C/^{3}H$ ratio 0.87 for Phe-tRNA^{cont}; 0.83 for Phe-tRNA^{ox-red}) as expected. Therefore, it is evident from these results that Phe-tRNA^{ox-red} is a very poor acceptor as compared to its equivalent control.

In order to test the effect of oxidation-reduction on tRNA more rigorously and to avoid any possible complication produced by side effects of ribosome binding, a simpler system was used. CACCA(Phe) and CACCA^{ox-red}(Phe) were isolated from their respective Phe-tRNAs after hydrolysis with T_1-RNase, and were used in the "fragment reaction" as acceptors with 70S ribosomes in the presence of alcohol (Monro and Marcker, 1967). This assay is thought to be the most direct and convenient way of investigating the catalytic center of peptidyl transferase. As in the preceding experiment, N-Ac-Phe-tRNA was the peptide donor. The results (Figure 16) show clearly that the oxidized-reduced fragment was completely inactive.

Table IX

Products Formed by Ribosome-bound N-acetyl-Phe-tRNA with Phe-tRNA^{ox-red} and Phe-tRNA^{cont}

Addition to Second Incubation	Ribosome-bound [^{14}C]Phe-tRNA (pmoles)	Pmoles of Products Formed		
		N-acetyl-Phe	N-acetyl-Phe-Phe [^{3}H]	N-acetyl-Phe-Phe [^{14}C]
a. None	--	25.55	0.048	0.055
b. Phe-tRNA^{cont}	19.2	9.75	7.42	6.48
c. Phe-tRNA^{ox-red}	14.5	24.1	0.77	0.64

The first reaction mixture contained (in 1.5 ml) 50 mM Tris-chloride, pH 7.4, 10 mM Mg)OAc)$_2$, 160 mM NH_4Cl, 1 mM DTT, 30 A_{260} units of poly U, 67.2 A_{260} units of ribosomes and 0.4 mM neutralized tetracycline. To this mixture were added 660 pmoles of N-acetyl[^{3}H]Phe-tRNA (200 mc/mmole). After incubation for 25 minutes at 35° C the sample was chilled and layered over 10% sucrose in 10 mM Tris-chloride, pH 7.4, 10 mM $Mg(OAc)_2$, 160 mM NH_4Cl, and 1 mM DTT and centrifuged at 150,000 x g for 2.5 hours in the Spinco 65 Ti rotor. The resulting pellet was rinsed with the buffer and suspended in 0.15 ml of the above buffer. Under these conditions, 56% of the input N-acetyl-phe-tRNA was bound to the ribosomes as judged by the Millipore filter technique ( ). This is assumed to be at the P site since 86% was releasable by subsequent incubation with puromycin in the presence of fusidic acid.

The second incubation mixture (0.1 ml) contained 50 mM Tris-chloride, pH 7.4, 100 mM NH_4Cl, 10 mM DTT, 80 mM KCl, 10 mM $Mg(OAc)_2$, 10 µg Poly U, and 3.9 A_{260} units of ribosomes containing 24.6 pmoles of bound N-acetyl-[^{3}H2Phe-tRNA. Reactions were started by adding 35 and 32 pmoles of [^{14}C]-Phe-tRNA^{cont} and [^{14}C]Phe-tRNA^{ox-red} respectively and incubating for 10 minutes at 35° C. The amount of [^{14}C]Phe-tRNA bound to the ribosomes under the above conditions was determined by the Millipore filter assay ( ). In a duplicate series, the reaction mixture was chilled at the end of the incubation and layered over 10% sucrose-buffer as above and centrifuged at 50,000 rpm for 10 hours. The pellet was resuspended in 30 µl of 0.3 KOH and incubated for 2 hours at 37° C. After neutralizing with $HClO_4$, the suspension was kept at 0° for 10 minutes and the precipitate of $KClO_4$ centrifuged off. The supernatant was applied to Whatman 3 MM paper with appropriate standards (N-acetyl-Phe, N-acetyl-Phe-Phe) and analyzed by electrophoresis in 0.025 M ammonium acetate, pH 6.8, at 2200 volts (44 v/cm) for 3 hours. The standards were located by their fluorescence at liquid nitrogen temperature under a UV lamp. The radioactive products were detected by cutting the paper into 1-cm strips and counting under 10 ml of toluene scintillation fluid using standard double label counting techniques for [^{14}C] and [^{3}H]. Under these conditions, N-acetylated produced moved to the anode and were well separated from each other. The amount of the product formed was determined from the amount of ^{14}C and ^{3}H label present at the position of N-acetyl-Phe-Phe (Figure 15).

Figure 15

Paper electrophoretic analysis of the reaction products formed by Phe-tRNA^{cont} and Phe-tRNA^{ox-red} with N-Acetyl-Phe-tRNA

Reaction components, conditions and the procedure are described in the legend of Table IX. The position of standards is indicated by the arrows.

[^{3}H] dashed bar

[^{14}C] closed bar

(a) N-acetyl[^{3}H]Phe-tRNA + addition

(b) N-acetyl[^{3}H]Phe-tRNA + [^{14}C]Phe-tRNA^{cont}

(c) N-acetyl[^{3}H]Phe-tRNA + [^{14}C]Phe-tRNA^{ox-red}

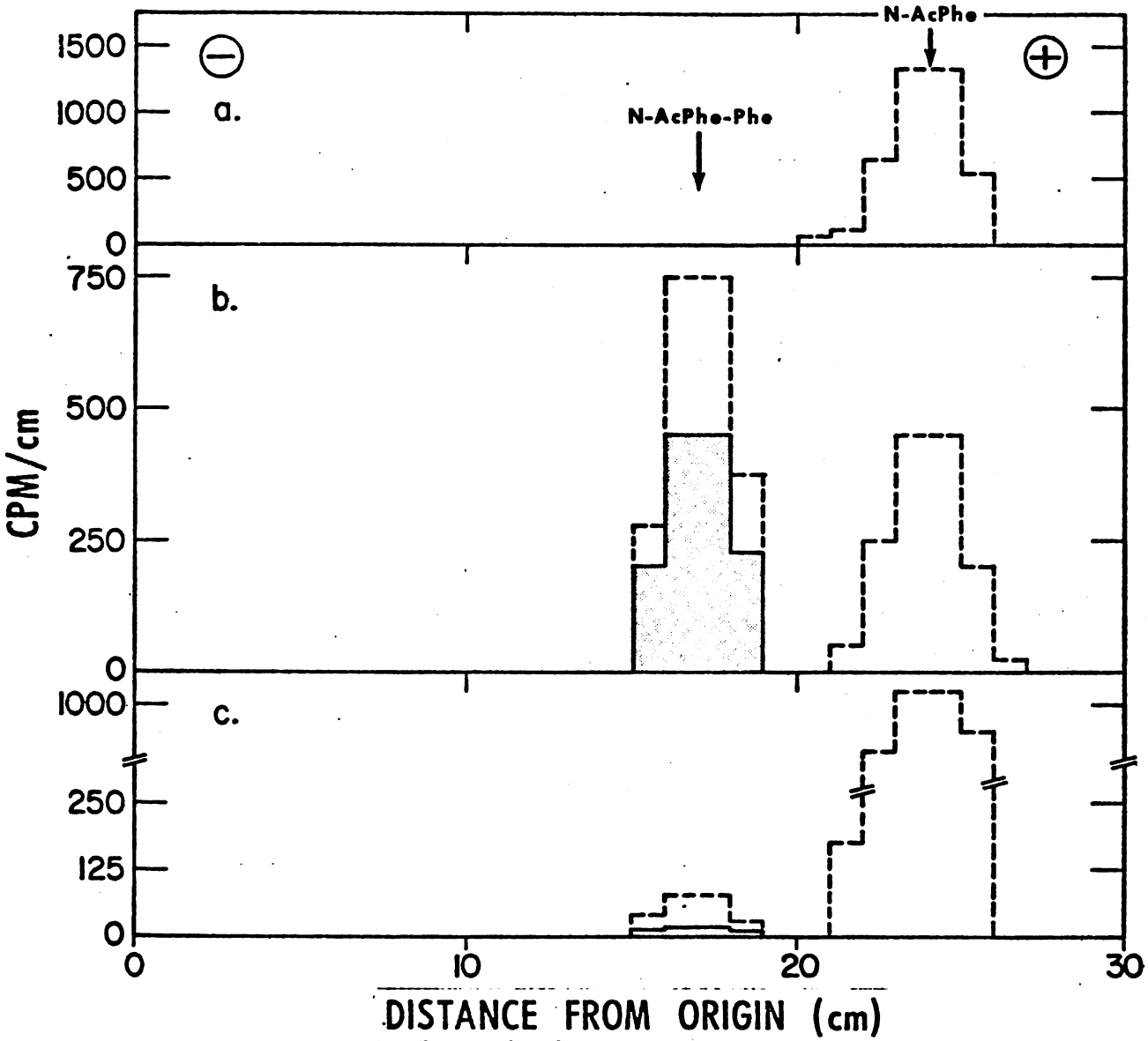
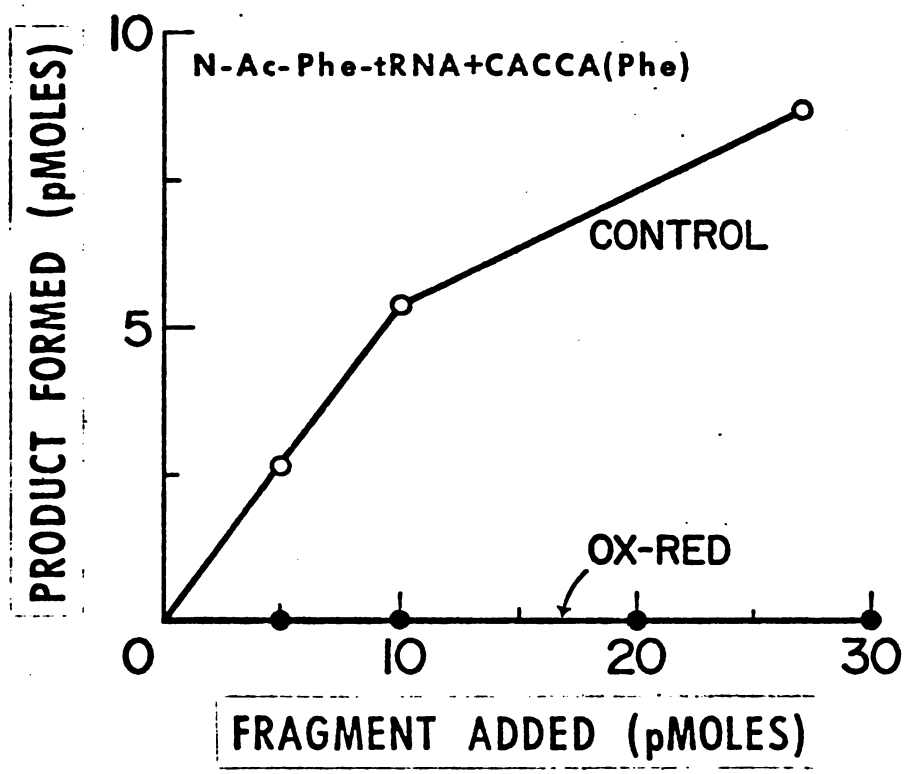

N-AcPhe
N-AcPhe-Phe
a.
b.
c.
1500
1000
500
0
750
500
250
0
1000
250
125
0
0
10
20
30
CPM/cm
DISTANCE FROM ORIGIN (cm)

Figure 16

Formation of N-acetyl-Phe-Phe from N-acetyl-Phe-tRNA and Oxidized-reduced CACCA(Phe).

50 µl of reaction mixture contained (before methanol addition), 0.06 M Tris-HCl, pH 7.4, 0.4 M KCl, 0.02 M $Mg(OAc)_2$, 4.0 A_{260} of activated 70S ribosomes, and 40 pmole of N-acetyl[^{3}H]Phe-tRNA. Acceptor fragments at the indicated concentrations were added last and the reaction initiated by the addition of 25 µl of methanol. After incubation at 35° C for 15 minutes, 3 ml of cold 5% TCA was added to stop the reaction and the mixture kept at 0° C for 10 minutes. The precipitated RNA was collected on a Millipore filter, washed 3 times with 3 ml portions of cold 5% TCA, dissolved in 10 ml of Bray's solution (Bray, G.A., 1960) and counted. The amount of peptide synthesized was determined as the difference between precipitable [^{3}H] radioactivity after incubation with and without added acceptor, since both CACCA([^{14}C]Phe) (Pestka, S. 1970) and the product CACCA(Ac[^{3}H]Phe[^{14}C]Phe) are soluble in cold 5% TCA. This was verified by the failure to find any TCA-precipitable [^{14}C]Phe radioactivity at the end of the reaction.

○ , $CACCA^{cont}$(Phe); ● , $CACCA^{ox-red}$(Phe).


PRODUCT FORMED (pMOLES)
10
5
0
N-Ac-Phe-tRNA+CACCA(Phe)
CONTROL
OX-RED
10
20
30
FRAGMENT ADDED (pMOLES)

c. Acceptor site activity of synthetic $Ado^{ox-red}(Phe)$, Ado(Phe) and puromycin on 70S and 50S ribosomes

In order to simplify the chemical nature of the acceptor further, the activity of $Ado^{ox-red}(Phe)$ was tested. Because of the limited amount of biologically synthesized material available and the high K_m expected for such substrates (Fahnestock et al., 1970) (see also Table X) it was not possible to do so with material isolated from Phe-tRNA. Therefore, the chemical synthesis of $Ado^{ox-red}(Phe)$ was carried out (Chapter II.B.10). As a control, Ado(Phe) was also synthesized by the same general method. Although the chemically synthesized product was expected to be inactive by analogy with the fragment CACCA(Phe) it surprisingly showed good activity in the N-Ac-Phe-tRNA-poly U-70S ribosomes assay (Figure 17). The time course for peptide formation at 35^{o} C is shown in Figure 17A for $Ado^{ox-red}(Phe)$, Ado(Phe) (both chemically synthesized) and for puromycin, each at 0.1 mM concentration. In each case the reaction initially proceeds surprisingly fast and then slows down. It does not reach a plateau even at 40 minutes. It is clear that Ado(Phe) is the most active acceptor followed closely by its analog, puromycin. As demonstrated in the figure, the difference was most noticeable at initial times. Similar results were reported earlier by Cerna et al., (1970). $Ado^{ox-red}(Phe)$ was less active, 40-50% compared to Ado(Phe). The concentration dependence curve (Figure 17B) confirms the higher activity of Ado(Phe) versus $Ado^{ox-red}(Phe)$. As seen in the figure, with Ado(Phe) and puromycin, maximum peptide formation took place at approximately 50 μM acceptor whereas with $Ado^{ox-red}(Phe)$ about a seven-fold higher concentration was

Figure 17

Extent of formation of N-acetyl-Phe Peptide from N-Acetyl-phe-tRNA and synthetic A^{ox-red} (Phe), A(Phe) and Puromycin

A. Assays were performed as described in Chapter II.D.1.a. except that 3.2 A_{260} units of the activated ribosomes, 50 pmoles of N-acetyl-[^{3}H]phe-tRNA and 0.1 mM acceptor substrates were used as indicated.

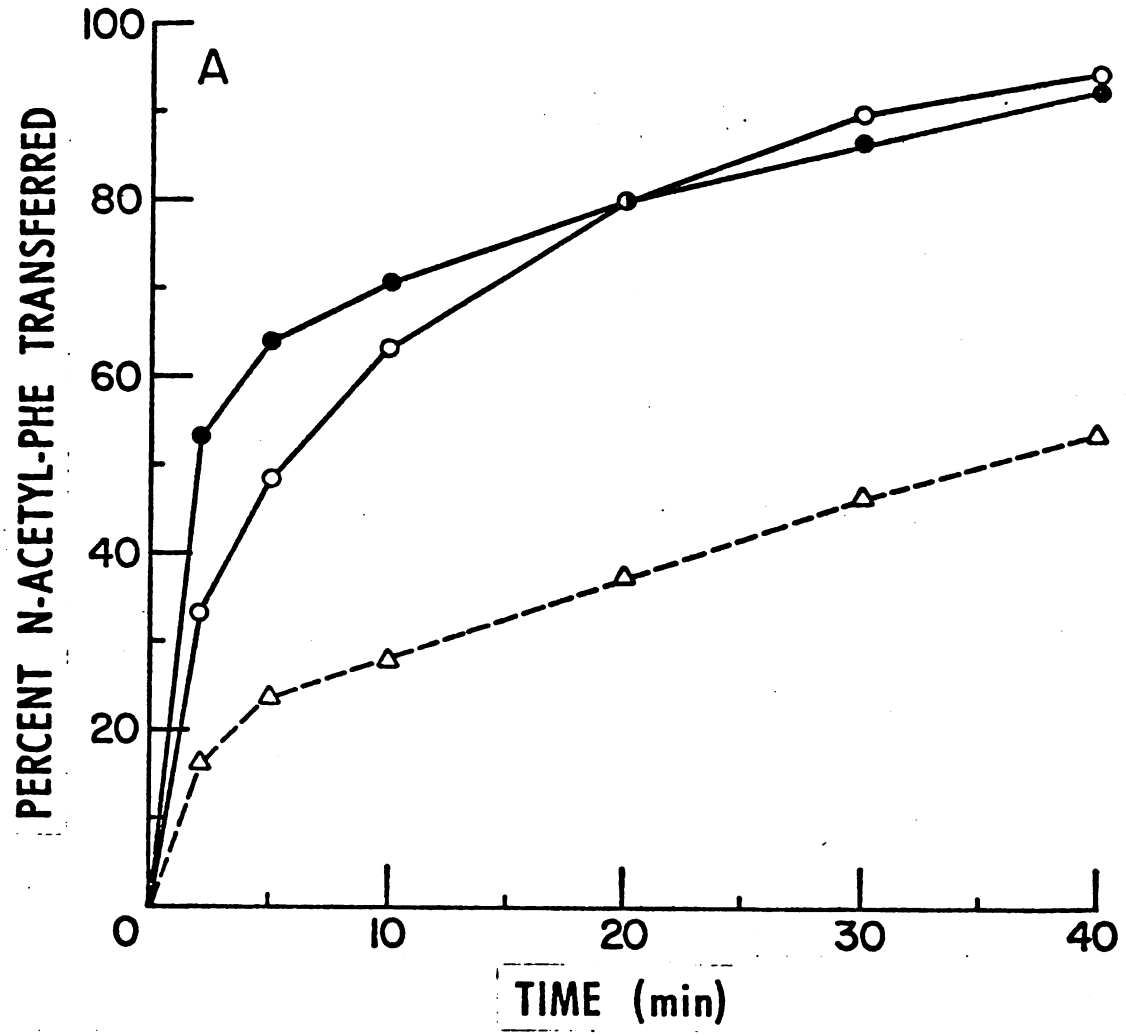
B. Concentration Dependence:

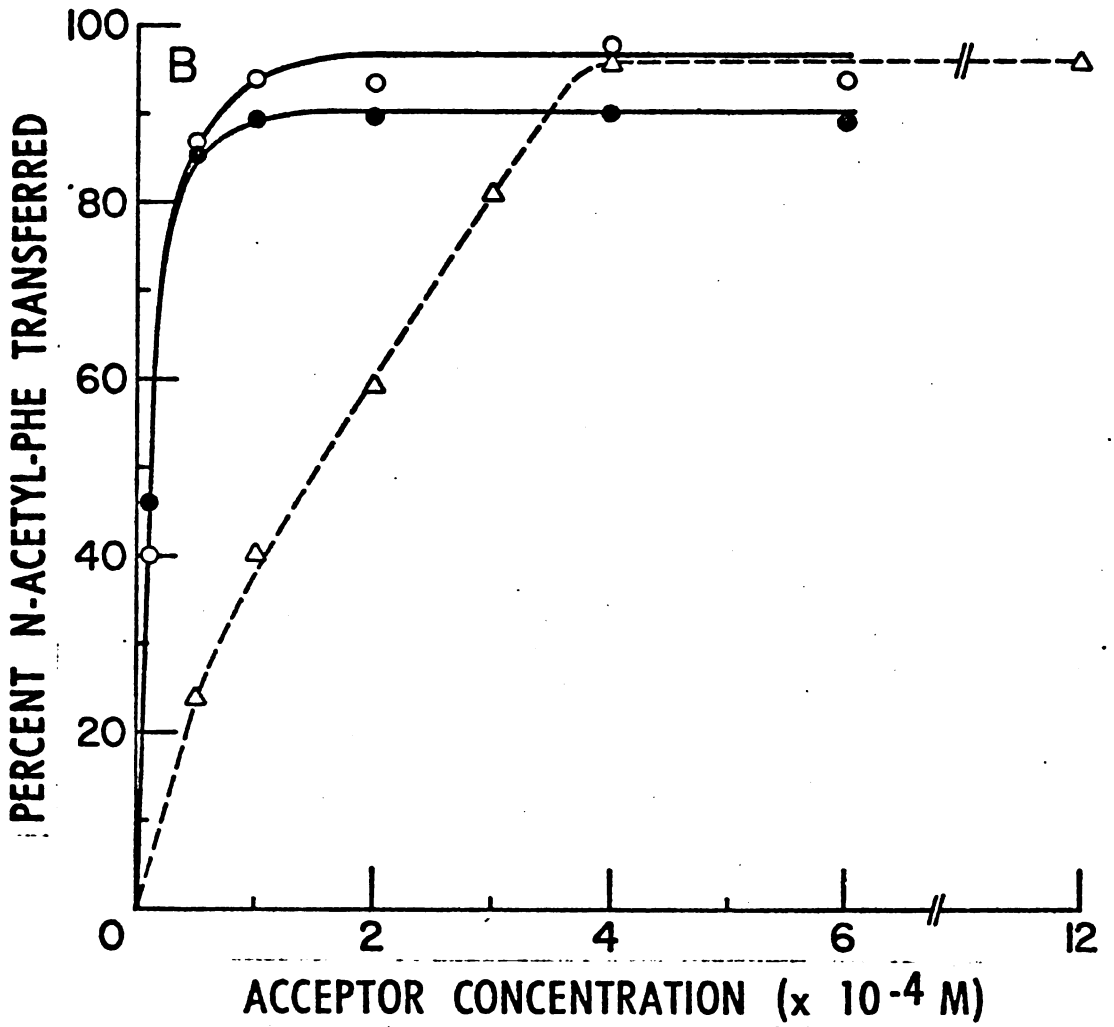
The reaction mixture (0.05 ml) was as in part A except that 33 pmoles of N-acetyl-[^{3}H]phe-tRNA and acceptor substrates at concentrations designated in the figure were used. Incubation was for 40 minutes at 35° C, and the mixture was processed for analysis as in part A above.

△ Synthetic A^{ox-red}(phe)

● Synthetic A(phe)

○ Puromycin


A
100
80
60
40
20
0
10
20
30
40
PERCENT N-ACETYL-PHE TRANSFERRED
TIME (min)


B
100
80
60
40
20
0
2
4
6
12
PERCENT N-ACETYL-PHE TRANSFERRED
ACCEPTOR CONCENTRATION (x 10-4 M)

required to achieve the same level of peptide bond formation. Ado^{ox-red}(Phe) was also active when tested in the 50S subunit-methanol assay (Figure 18). Attainment of the same maximum level of product formation at higher concentration (Figure 17B) rules out the possibility of the presence of an inhibitor in the Ado^{ox-red}(Phe) preparation and suggests that true peptide bond formation was being studied. Since the synthetic acceptor Ado^{ox-red}(Phe) 0.082 mC/mM) was largely extracted into ethyl acetate under the conditions of the assay, it was necessary to eliminate the radioactivity contributed by it in order to measure peptide bond formation. This was done by counting the ethyl acetate extractable radioactivity for [^{3}H] with exclusion of [^{14}C] and by including a control with each concentration of Ado^{ox-red}(Phe) in which the donor N-Ac[^{3}H]Phe-tRNA was omitted. A separate control without acceptor was also subtracted. At the maximum concentration of acceptor, the total blank value was around 20% of the experimental counts when peptide bond formation took place.

d. Identification of products

N-Ac-Phe-puromycin, N-Ac-Phe-Phe-adenosine and N-Ac-Phe-Phe-adenosine^{ox-red} are the expected products of the respective peptide transfer reactions of N-Ac-Phe-tRNA with puromycin, Ado(Phe) and Ado^{ox-red}(Phe) respectively (Figures 17 and 18). To prove this, the products were identified by high voltage electrophoresis at pH 3.5. (Figure 19). In this system, N-Ac-Phe-Phe-adenosine and N-Ac-Phe-Phe-adenosine^{ox-red} moved to the same position toward the cathode and slightly behind N-Ac-Phe-puromycin (Cerna et al., 1970). Moreover, mild alkaline

Figure 18

Transfer of N-acetyl-Phe residue from N-acetyl-Phe-tRNA to acceptor substrates, synthetic A(Phe), A^{ox-red}(Phe) and puromycin on 50S ribosomes.

Assays were performed as described in Chapter II.D.1.b except that substrate concentrations were used as indicated and 50 pmoles of N-acetyl[^{3}H]Phe-tRNA was added. Incubation was at 22° C for 45 minutes. Blanks without acceptor have been subtracted from each value.

△ , synthetic A(Phe)

○ , puromycin

● , synthetic A^{ox-red}(Phe)

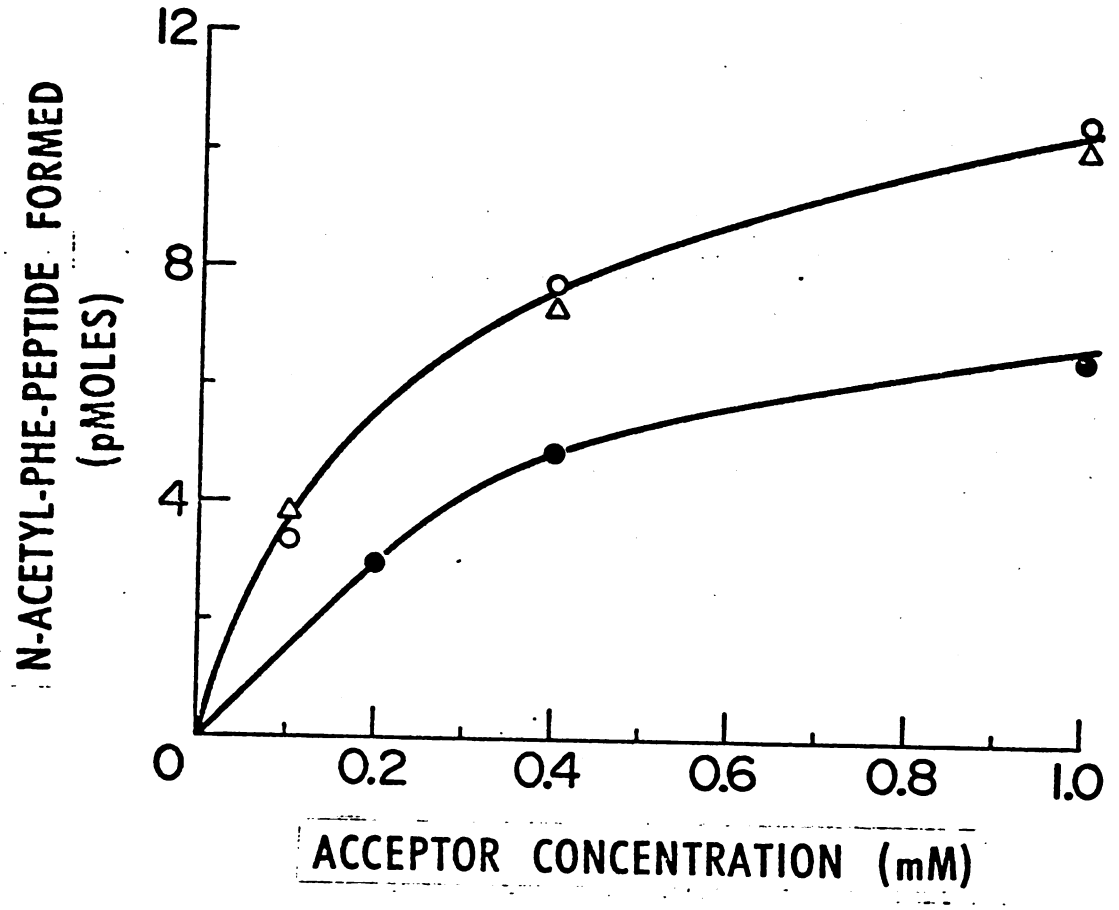

N-ACETYL-PHE-PEPTIDE FORMED
(pMOLES)
12
8
4
0
0.2
0.4
0.6
0.8
1.0
ACCEPTOR CONCENTRATION (mM)

Figure 19

Electrophoretic analysis of the reaction products formed by transfer of the N-acetyl-Phe residue from N-acetyl-Phe-tRNA to the acceptor substrates, puromycin, A(Phe) and A^{ox-red}(Phe)

The reaction mixture described in Figure 17 contained (in 0.1 ml), 100 pmoles of N-acetyl[^{3}H]Phe-tRNA, 10 A_{260} units of ribosomes and 400 μM acceptor as indicated below. Incubation was for 60 minutes at 35° C. The reaction was terminated and the product extracted into 5 ml of ethylacetate as described in Chapter II.D.1.a. and evaporated to a small volume. Samples to be hydrolyzed were evaporated to dryness, dissolved in 20 μl of 0.2 N NaOH and incubated for 20 minutes at 37° C. Samples were then neutralized with 2 μl of 2 N HCl, applied to Whatman 3 MM paper and the reaction products were isolated by electrophoreses in 5% acetic acid and 0.5% pyridine (v/v), pH 3.5 at 2000 volts (40 volts/cm) for 3.5 hours. Radioactivity on paper was located by immersing 1-cm strips in 10 ml of toluene scintillation fluid and counting in a liquid scintillation spectrometer. Positions of N-Acphephe and NACphe are indicated by arrows.

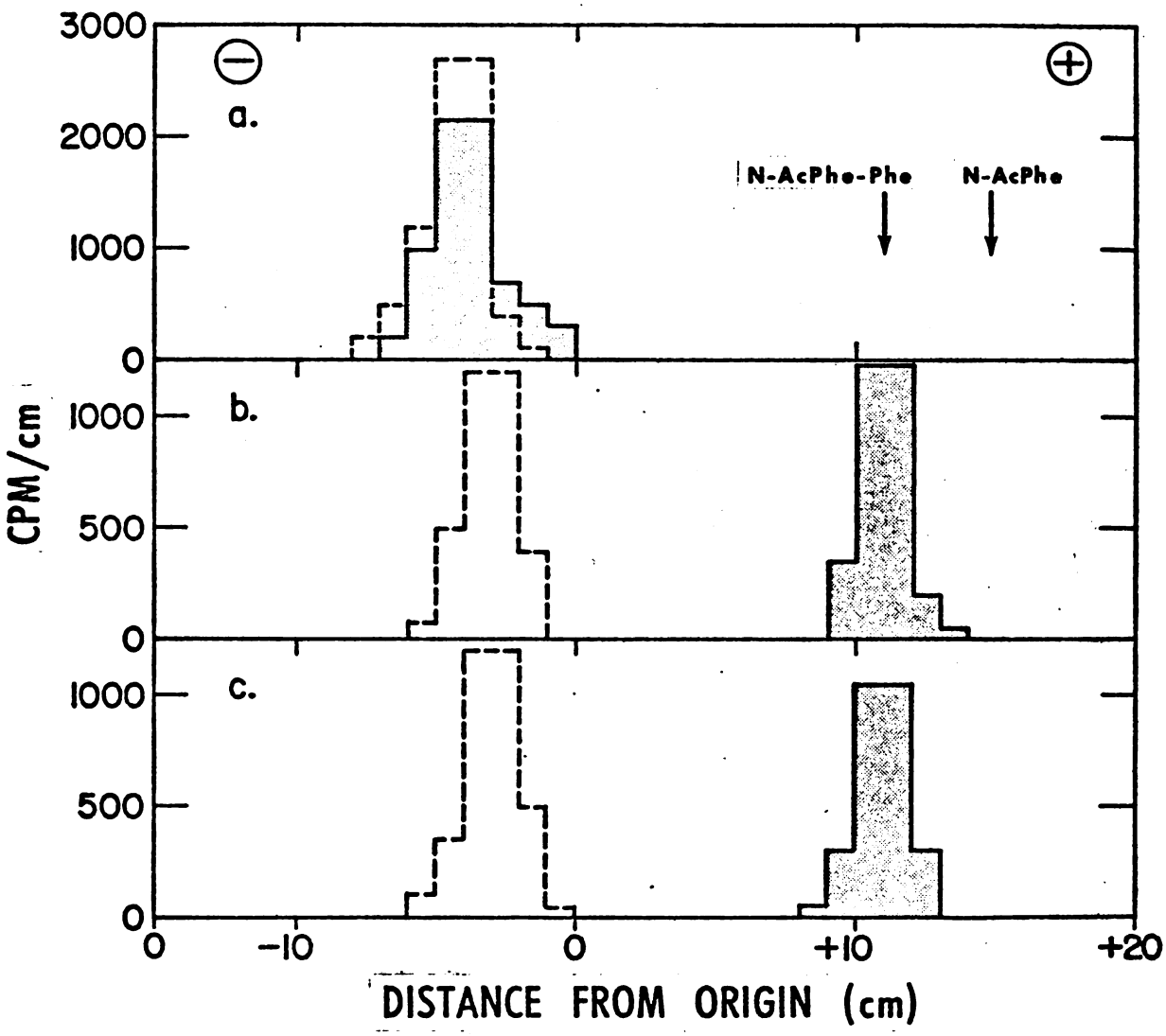
dashed bar, unhydrolyzed

closed, after alkaline hydrolysis

Panel (a) - Puromycin

Panel (b) - Ado(Phe)

Panel (c) - Ado^{ox-red}(Phe)


3000
2000
1000
0
a.
N-AcPhe-Phe
N-AcPhe
1000
500
0
b.
1000
500
0
c.
CPM/cm
0
-10
0
+10
+20
DISTANCE FROM ORIGIN (cm)

hydrolysis of these products (except for N-Ac-Phe-puromycin which is not hydrolyzed under these conditions) gave rise to the acetylated dipeptide, N-Ac-Phe-Phe. Clearly, the ethyl acetate extraction procedure is a valid measure of Ado^{ox-red} (N-Ac-Phe-Phe) formation.

3. Kinetics of peptide bond formation

a. Kinetics of peptide bond formation using synthetic Ado^{ox-red}(Phe) and Ado(Phe) on 70S ribosomes

To study the activity of synthetic Ado^{ox-red}(Phe) more rigorously the kinetics of peptide formation was followed using 70S washed ribosomes. Figure 20A shows the time dependence of peptide formation for the acceptor compounds as measured by ethyl acetate extractable radioactivity. At 600 μM each of puromycin and Ado^{ox-red}(Phe), peptide formation was linear up to 2 minutes and at least 6 minutes respectively; with Ado(Phe) at 200 μM, it was linear up to 4 minutes. The initial velocity of peptide bond formation as a function of acceptor concentration is depicted in Figure 20B for synthetic Ado^{ox-red}(Phe), its control, and for puromycin as a reference standard. It is clear that while oxidation-reduction has reduced the affinity of Ado(Phe) for peptidyl transferase, strong activity is still present. K_m values were calculated from the S/V versus S plots of the data (Figure 21) and tabulated in Table X. Note that while the V_{max} is not affected, the K_m has been increased six times by the oxidation-reduction cleavage.

b. Activity and kinetics of synthetic acceptor substrates on polysomes.

In view of the difference in peptide forming ability between chemically synthesized Ado^{ox-red}(Phe) and biosynthetic CACCA^{ox-red}(Phe),

Figure 20

Rate of formation of N-acetyl-Phe-peptide from N-acetyl-Phe-tRNA and synthetic Ado-(Phe), Ado^{ox-red}(Phe) and puromycin on washed 70S ribosomes

A. Time course:

The incubation mixture (0.05 ml) was as described in Chapter II.D.1.a. except that 2 A_{260} units of activated 70S ribosomes, and 50 pmoles of N-acetyl[^{3}H]Phe-tRNA were used. Reaction was started by adding acceptor substrates at the concentrations shown below. After incubation at 35° C for the indicated time, the reaction was terminated by the procedure described in Chapter II,D.1.a. and the product processed as described there. Radioactivity of a blank incubation without acceptor was substracted. Counts were corrected for the sample volume and the amount of peptide formed was expressed in pmole.

● , puromycin 600 µM

○ , synthetic Ado-(Phe) 200 µM

▲ , synthetic Ado^{ox-red}(Phe) 600 µM

B. Concentration dependence of initial rate:

Components of the reaction mixture are described in A. Reaction was initiated by adding acceptor substrates at concentrations shown in the figure. The mixture was incubated at 35° C for the times indicated below and processed as in A. After correction for the sample size, results were expressed as pmoles product

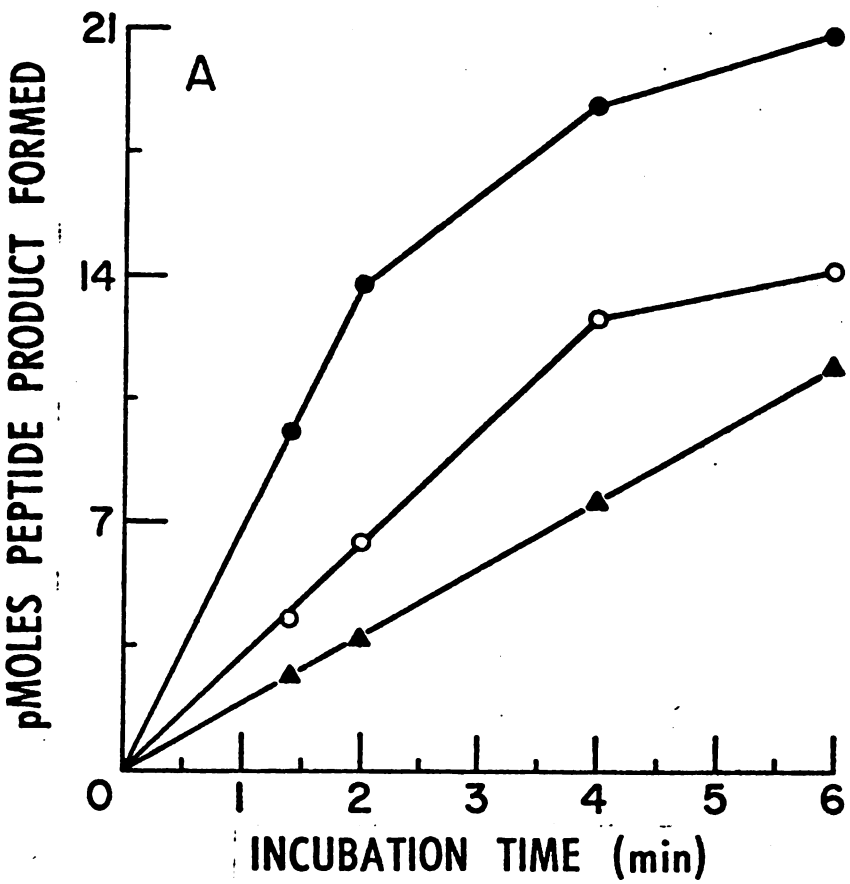
formed per minute per A_{260} ribosomes.

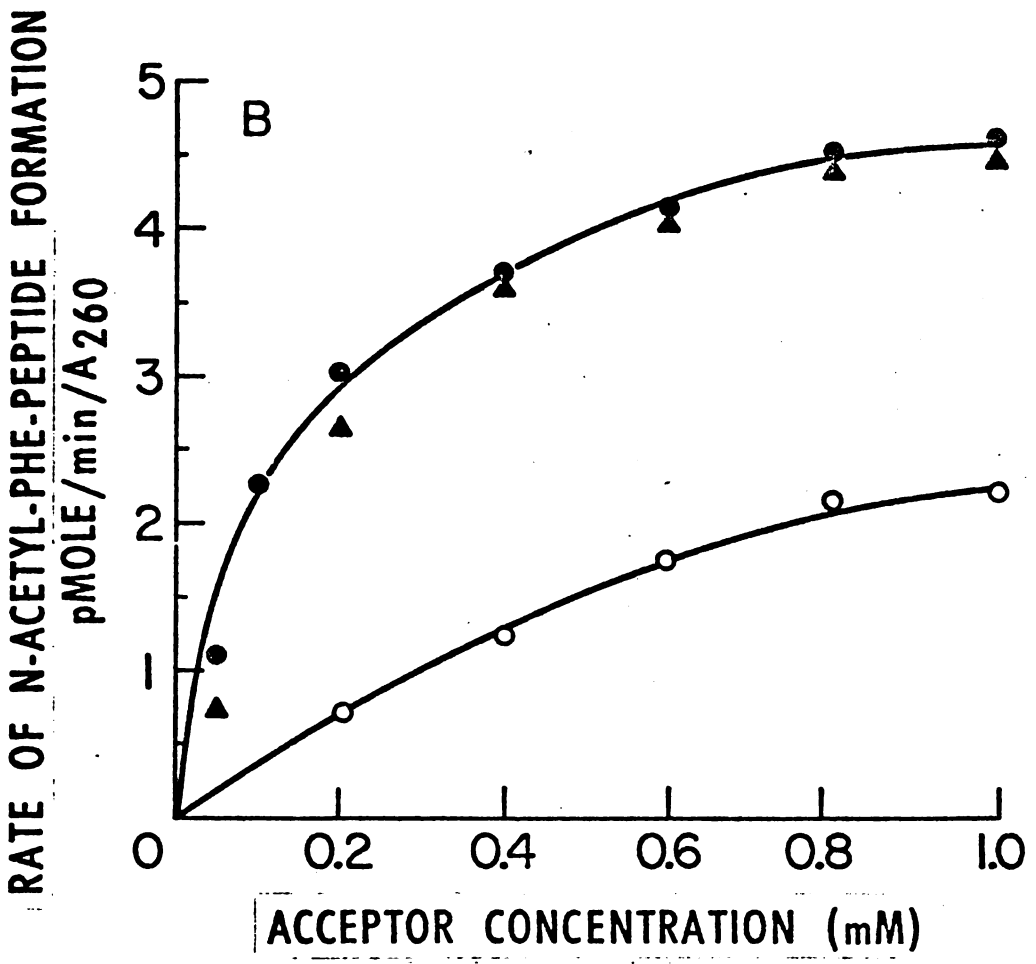
▲ , Ado(Phe, incubation time was 3 minutes

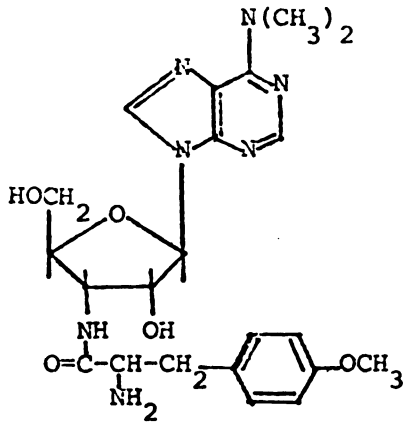
○ , Ado^{ox-red}(Phe), incubation time was 3 minutes

● , puromycin, incubation time was 1.5 minutes

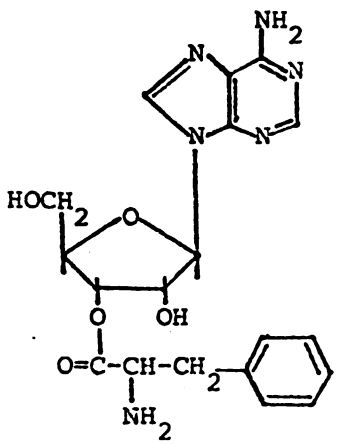
C. Structure of puromycin, L-phenylalanyl 3'-adenosine, and L-Tyrosyl 3'=Adenosine.


A
21
14
7
0
1
2
3
4
5
6
pMOLES PEPTIDE PRODUCT FORMED
INCUBATION TIME (min)

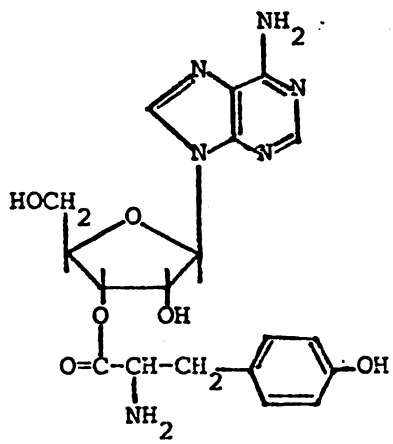

RATE OF N-ACETYL-PHE-PEPTIDE FORMATION
pMOLE/min/A260
B
5
4
3
2
1
0
0.2
0.4
0.6
0.8
1.0
ACCEPTOR CONCENTRATION (mM)



PUROMYCIN



L-PHENYLALANYL 3'-ADENOSINE



L-TYROSYL 3'-ADENOSINE

Figure 21

Determination of K_m for the formation of N-acetyl-Phe-peptide from N-acetyl-Phe-tRNA and synthetic A(Phe), A^{ox-red}(Phe) and Puromycin on washed 70S ribosomes

S/V versus S plot was drawn from the data of Figure 20. V_{max} was calculated from the slope of the curve and the K_m from the ratio of intercept to slope as described.

● , puromycin; ○ , Ado(Phe); Δ , Ado^{ox-red}(Phe)

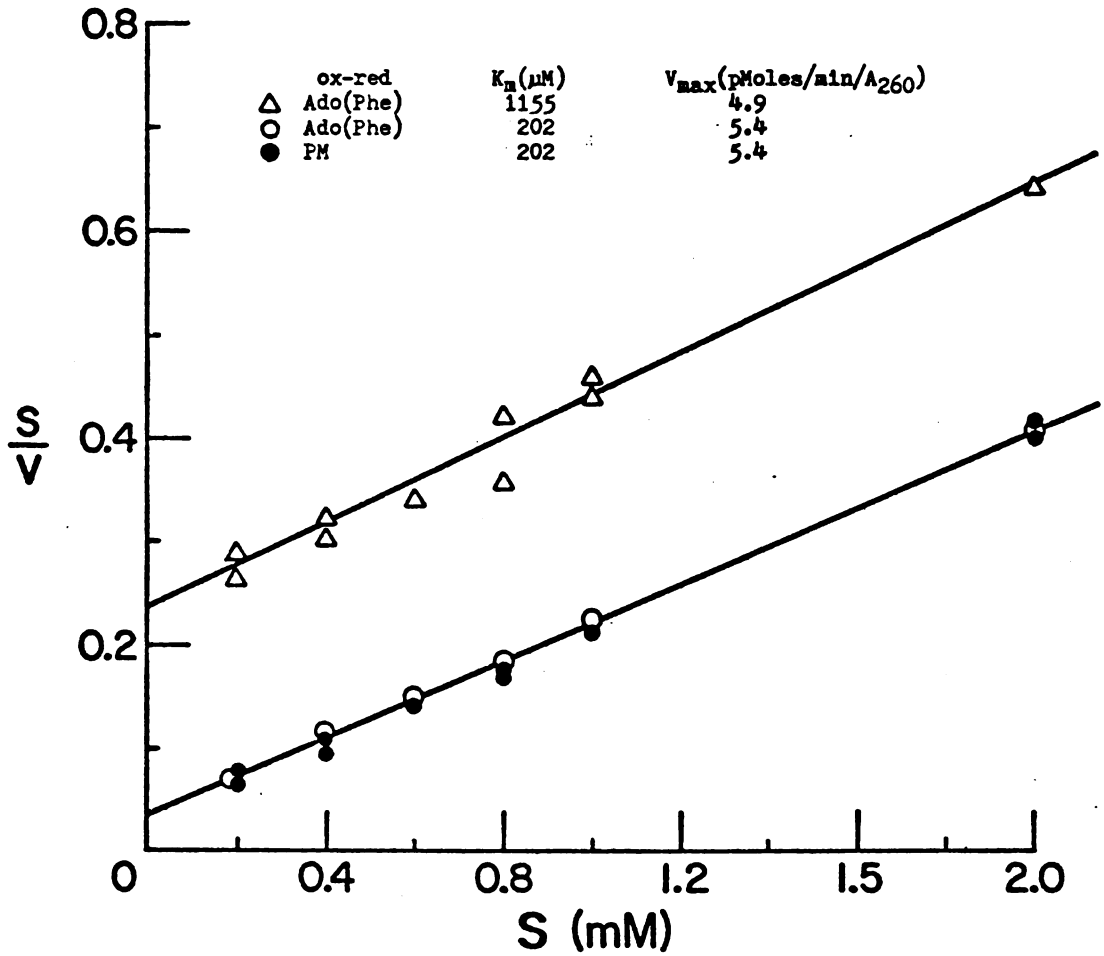

0.8
0.6
0.4
0.2
0
S/V
ox-red
Ado(Phe)
Ado(Phe)
PM
K_m(μM)
1155
202
202
V_{max}(pMoles/min/A_{260})
4.9
5.4
5.4
0.4
0.8
1.2
1.5
2.0
S (mM)

TABLE X

Summary of Kinetic Constants of Acceptor Substrates for Peptide Bond Formation on Ribosomes

Acceptor Substrate	Ribosomes	
	K_m	V_{max}
	μM	pmole/min/A_{260}
Puromycin	202	5.0
Synthetic Ado(Phe)	202	5.4
Synthetic Ado^{ox-red}(Phe)	1155	4.9

Kinetic constants were obtained from the $\frac{S}{V}$ versus S plots shown in Figure 21.

it became essential to test Ado^{ox-red}(Phe) derived from Phe-tRNA^{ox-red}. Since it could not be done with washed ribosomes, the peptidyl transferase system used so far in this study, due to the larger amount of substrate needed for detectable reaction, the more sensitive polysome system was used. Polysomes from E. coli were recently shown by Pestka et al. (1970), to have a 75-fold lower K_m for acceptor substrates like puromycin. In this system, naturally synthesized polypeptidyl-tRNA on polysomes reacts with added labeled acceptor substrates producing peptidyl-acceptors which due to their larger peptidyl moiety are insoluble in 5% cold TCA. Figure 22 shows the dependence of the extent of peptide bond formation upon the concentration of Ado^{ox-red}(Phe), Ado(Phe), both derived from their respective Phe-tRNAs, and of puromycin. In this system, Ado^{ox-red}(Phe) was totally inactive, both at 35° C and and at 25° C as expected from the inactivity of CACCA^{ox-red}(Phe) in the washed ribosome-methanol assay system. Ado(Phe) and puromycin were both active. In view of the above result, chemically prepared Ado^{ox-red}(Phe) was also tested in the polysome system. Unfortunately, the specific radioactivity of the available Ado^{ox-red}(Phe) was not sufficient to allow direct estimation of the reaction product. Since the donor peptidyl-tRNA was unlabelled, the acceptor must be labelled in order to obtain a measurable product.

Instead, an attempt was made to detect the activity indirectly. The effect of synthetic Ado^{ox-red}(Phe) and Ado(Phe) was checked on the rate of peptidyl[^{3}H]puromycin formation (data not shown). It was observed that both compounds inhibited peptidyl[^{3}H]puromycin formation and Ado^{ox-red}(Phe) was less active than Ado(Phe). Although inhibition of peptidyl[^{3}H]puromycin formation was detected with both Ado(Phe) and Ado^{ox-red}(Phe) preparations, one can be sure only in the case of Ado(Phe)

Figure 22

Extent of peptide bond formation between peptidyl-tRNA on polysomes and Ado^{ox-red}(Phe) and Ado(Phe)

Ado^{ox-red}(Phe) and Ado(Phe) were obtained by means of pancreatic RNase digestion of Phe-$tRNA^{ox-red}$ and Phe-tRNA respectively, as described in Chapter II.B.8. The assay conditions were as described in Chapter II.D.2.b.

○ ● , [^{3}H]puromycin

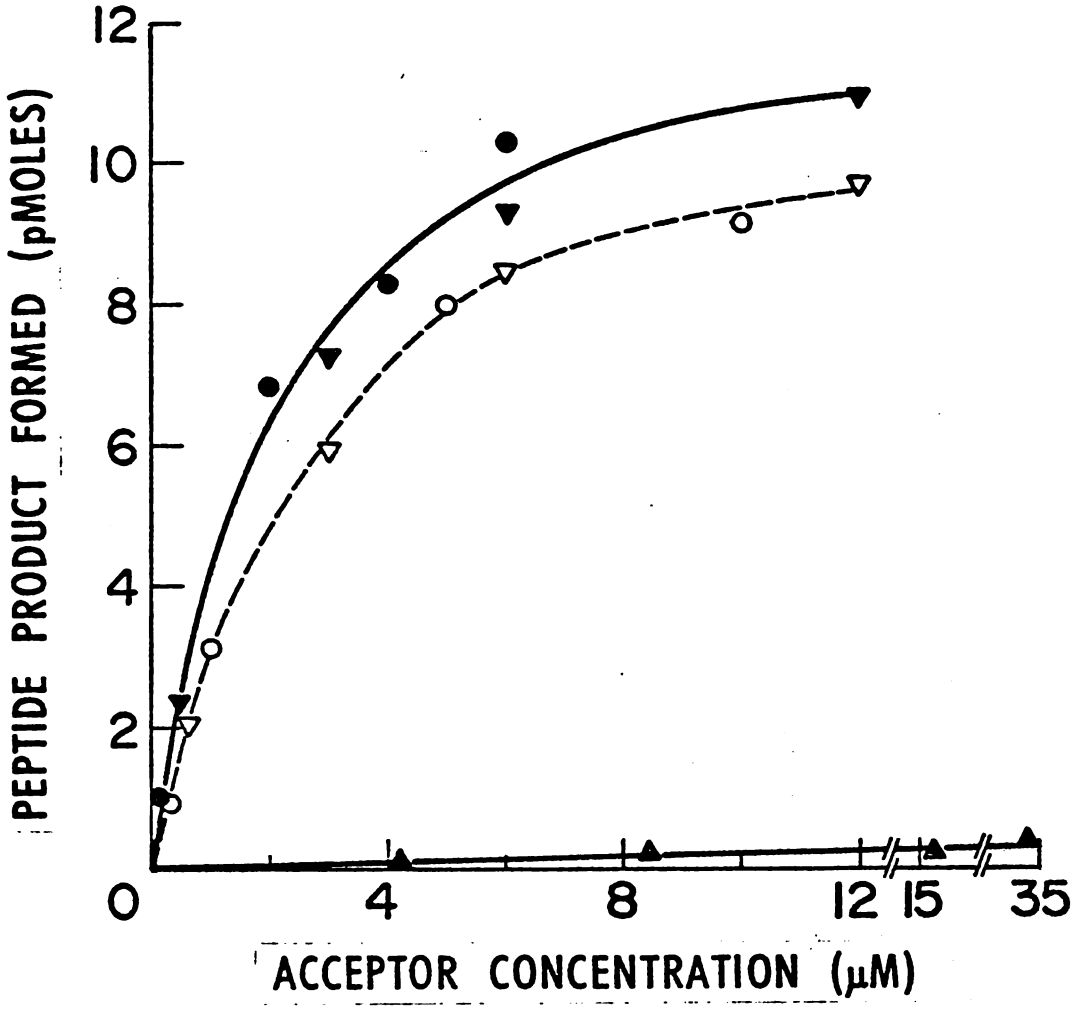
▲ , Ado^{ox-red}[^{3}H-phe]

▽ ▼ , A-[^{14}C-Phe]

solid symbols, solid line, at 35° C

open symbols, dashed line, at 25° C

▲ , both at 25° C and 35° C


PEPTIDE PRODUCT FORMED (pMOLES)
12
10
8
6
4
2
0
4
8
12
15
35
ACCEPTOR CONCENTRATION (μM)

that the inhibition shown by it was due to peptide product formation (i.e., peptidyl-Phe-Ado, see Figure 22). The same cannot be said for Ado^{ox-red}(Phe) since it was not shown by an independent experiment that Ado^{ox-red}(Phe) formed peptidyl-Phe-Ado^{ox-red} on polyribosomes although N-acetyl-Phe-Phe-Ado^{ox-red} formation on washed ribosomes is evident (see figures 17-20). The possibility that synthetic Ado^{ox-red}(Phe) acted in this instance only as a true inhibitor, cannot be ruled out completely. Therefore, the inhibition of peptidyl[^{3}H]puromycin formation by Ado^{ox-red}(Phe) is not considered with due emphasis.

4. Summary of the reactivity of various substrates in peptide bond formation.

In summary, a variety of substrates have been used in the peptidyl transferase reaction on ribosomes and polysomes in order to understand the effect produced by the oxidation-reduction of $tRNA^{phe}$. Table XI shows all the substrates, the activity of which were tested at both the P-site and A-site. It is interesting to note that enzymatically aminoacylated $tRNA^{ox-red}$ was inactive for the transfer reaction from both sites. Since it was not possible to narrow down the P-site activity test to mononucleotide level (the substrate needs at least 3 nucleotides) (Mercer and Symons, 1972; Monro et al., 1968), the A-site study was pursued to that level.

The results presented in this study show conclusively that the inability of Phe-$tRNA^{ox-red}$ to participate in the peptidyl transferase reaction is due to the chemical change brought about by cleavage of the $C_{2'}$, $C_{3'}$ bond only and is not due to any change in the inherent properties and arrangement of other bases in the tRNA molecule since the effect persisted down to the Ado(Phe) level. The difference in the reactivity between synthetic Ado^{ox-red}(Phe) and biologically derived Ado^{ox-red}(Phe) can only be reconciled by assuming that these two compounds differ in the exact position of the phenylalanyl moiety on adenosine (i.e., $C_{2'}$ or $C_{3'}$) and that only one is recognized by the peptidyl transferase system. This point will be further considered in the Discussion.

TABLE XI

Summary of the Reactivity of Various Oxidized-Reduced Substrates with Ribosome-bound Peptidyl Transferase

Substrates	Activity
Acceptors	
Phe-tRNA^{control}	Active
Phe-tRNA^{ox-red}	Inactive
CACCA^{control}(Phe)	Active
CACCA^{ox-red}(Phe)	Inactive
° Ado(Phe), synthetic	Active
° Ado^{ox-red}(Phe), synthetic	Active
* Ado(Phe) from phe-tRNA	Active
* A^{ox-red}(Phe) from Phe-tRNA^{ox-red}	Inactive
Donors	
N-Ac-Phe-tRNA^{cont}	Active
N-Ac-Phe-tRNA^{ox-red}	Inactive
CACCA^{cont}(N-Ac-Phe)	Active
CACCA^{ox-red}(N-Ac-Phe)	Inactive

Data are taken from the experiments shown in Figures 12-23. The experimental details are described therein. Except as indicated, assays were performed using washed ribosomes.

* Tested on polysomes only due to limited amount of substrate.

° Tested both on ribosomes and polysomes.

5. Rationale for peptide bond synthesis using aminoacyl-oligonucleotide.

It is known that during protein synthesis only codon-specified aminoacyl-tRNA binds to the A-site on ribosomes and interacts properly with the peptidyl-transferase center resulting in peptide bond formation. Since the concentration of total charged tRNA in E. coli (estimated to be 300-600 μM (Watson, 1965; Maaløe and Kjeldgaard, 1966; Lewis and Ames, 1972; Falk and Berg, 1970) is well above the concentration of puromycin (20 μM) lethal to permeable mutants of E. coli in vivo (Ennis, 1971), one is led to consider the nature of the molecular forces which must operate to prevent uncoded aminoacyl-tRNAs from reacting with nascent peptide chains in a manner analogous to the reaction with puromycin. Furthermore, it was shown a number of years ago (Takanami, 1964) that a mixture of AA-oligonucleotides of varying lengths (> 3), prepared by completely digesting aminoacylated unfractionated tRNA with T_1-RNase, was almost as effective as puromycin in removing nascent polypeptide chains from ribosomes. Therefore, it seemed apparent that some aspect of the structure of aminoacyl-tRNA as compared to small aminoacyl-oligonucleotides prevents access of the aminoacyl adenosine end of the tRNA to the peptidyl transferase catalytic center. Previous work in the field has not examined this question. It is well established that aminoacyl-adenosine can substitute for puromycin in vitro (Waller et al., 1966), and that substitution of the 5'-hydroxyl of the adenosine by phosphate or cytidylyl residues markedly enhances activity (Rychlik et al., 1967; Gottikh et al., 1970). However, despite the preparation and assay of an ingenious variety of puromycin analogs, no further study of the effect of oligonucleotide chain length has been carried out up to now.

As a first approximation to an analysis of this situation, peptide bond formation with aminoacyl-oligonucleotides of undefined chain length was undertaken to repeat and confirm Takanami's result (1964). Then to extend this work, the ability of several tyrosyl-oligonucleotides of defined chain length to act as acceptor substrates in peptide bond formation was also tested. The apparent K_m and V_{max} for the reaction were measured using both a model system reconstructed from purified components (N-acetyl-Phe-tRNA bound to salt-washed ribosomes) as well as a more physiological system consisting of washed polysomes carrying nascent peptidyl-tRNA.

6. Effect of RNase digests of aminoacyl-tRNA on nascent peptide chains

Nascent peptide chain-ribosome complexes were prepared as described in Chapter II.C.4. The polyphenylalanine system was chosen because polyphenylalanine once synthesized on ribosomes remains more tightly bound than polylysine (Traut and Monro, 1964), the usual alternative. Using these "labeled ribosomes" (3550 cpm/mg), the effect of an RNase digest of aminoacyl-tRNA was tested (Table XII). As controls, the effects of untreated aminoacyl-tRNA, T_1-RNase and pancreatic RNase were compared under similar conditions. As can be seen in the Table, the same concentration of a T_1-RNase digest of AA-tRNA caused significantly more (24% more than the AA-tRNA control) release of the label to the soluble fraction than either the pancreatic RNase digest (5%) or puromycin (18%). Increasing the concentration of the T_1-RNase digest gave even more (38%) release. The 20% release observed in the case of untreated aminoacyl-tRNA control was also noted with the two RNase controls and represents a blank value. These results are in qualitative agreement with Takanami's report (1964) who used the same assay method. The fact that the releasing effect of the T_1-RNase digest was much more marked than that of the pancreatic RNase digest seemed to suggest that the larger fragments produced by T_1-RNase were much more efficient acceptors for peptide bond formation than was aminoacyl-adenosine produced by pancreatic RNase. In order to investigate this further, a simpler assay was devised to examine the release of nascent polypeptide. Testing of various TCA concentrations showed that it was possible to preferentially precipitate unreacted polyphenylalanyl-tRNA with 0.1% trichloroacetic acid (TCA) while the product of the transfer reaction with aminoacyl-oligonucleotide or with aminoacyl-

TABLE XII

The Effect of RNase Digests of Mixed Aminoacyl-tRNA on the Nascent Polypeptide Chain of Ribosomes

ADDITIONS	CONCENTRATION[a] (M)	RADIOACTIVITY IN PELLET (cpm)	% RELEASE[b]
None	--	2523	0
Puromycin	2×10^{-4}	1560	38
AA-tRNA treated with T_1-RNase	2×10^{-4}	1411	44
	2×10^{-3}	1067	58
AA-tRNA treated with Pan-RNase	2×10^{-4}	1900	25
AA-tRNA	2×10^{-4}	2014	20
T_1-RNase	10 µg	1980	21
Pan-RNase	10 µg	2120	18

[a] Concentration of RNase treated aminoacyl-tRNA was estimated from the average specific activity of [^{3}H]aminoacyl-tRNA.

[b] % release was calculated from the loss of counts in the pellett.

The method is a modification of the procedure described by Takanami (1964). The reaction mixture contained in a final volume of 2 ml, 1.4 mg of "labelled ribosomes" (5000 cpm), prepared as mentioned in Chapter II.C.4., 60 mM KCl, 15 mM MgOAc, 50 mM Tris-acetate, pH 7.5, 0.1 mM spermine, and the additions as indicated in the Table. Incubations were for 10 minutes at 37° C. After chilling the tubes at 0°, 1.2 mg of carrier ribosomes were added and the mixture centrifuged at 100,000 x g for 2 hours. The supernate was carefully aspirated, the pellett was rinsed once with the buffer (50 mM Tris-acetate, pH 7.5, 60 mM KCl, 15 mM MgOAc)$_2$ and then suspended in 0.2 ml of the same buffer and acid-insoluble radioactivity remaining in the pellet was measured. 8 ml of cold 5% TCA was added to the pellet and after 10 minutes at 0° the precipitate was collected on a glass fiber filter (GF/C). The filter was dried for 30 minutes at 100° and the radioactivity counted under 10 ml of toluene scintillation fluid.

adenosine (e.g., polyphenylalanyl-AA-oligonucleotide or polyphenylalanyl-AA-adenosine) was soluble. This was determined by conducting an equivalent experiment using the centrifugation assay and comparing the results obtained with the 0.1% TCA method. The amount of product formed (i.e., loss of ribosome-bound cpm) was the same by both methods. Increasing the concentration of TCA resulted in increased precipitation of the reaction products. Therefore, the loss of precipitable radioactivity represented product formation. This assay could not be used for puromycin since reaction with polyphenylalanyl-puromycin was also found to be insoluble in 0.1% TCA. The m-cresol assay described by Traut and Monro (1968) could not be used for these experiments because whereas polyphenylalanyl-puromycin is soluble in m-cresol, the polyphenylalanyl-AA-oligonucleotide or polyphenylalanyl-AA-adenosine is not. Although the validity of this assay method primarily depends on the chain length of the peptidyl moiety, it could conveniently be used with the same batch of "labeled ribosomes." By this assay also, (Table XIII), the T_1-RNase digest was much more efficient (30% release at 200 μM) in stripping the nascent polypeptides than the pancreatic RNase digest (9% release at 200 μM). The same amount of release was observed with the untreated AA-tRNA control as was found by the centrifugation assay. Controls containing the same amount of digesting enzymes together with the reaction mixture minus AA-tRNA showed no effect. As mentioned above, puromycin in this assay system shows no apparent effect because the product also is precipitated in 0.1% TCA. The m-cresol assay showed that there was a 20% release by puromycin over the blank value found with AA-tRNA (data not shown).

The percentage release value is not necessarily proportional to the

TABLE XIII

Effect of RNase digests of aminoacyl-tRNA on the release of nascent polypeptides from ribosomes

ADDITIONS	CONCENTRATION* (M)	PERCENT RELEASE**
None	-	0
Puromycin	2×10^{-4}	2
	4×10^{-4}	5
AA-tRNA, T_1-RNase treated	2×10^{-4}	50
	1×10^{-3}	76
	2×10^{-3}	86
AA-tRNA, Pancreatic-RNase treated	2×10^{-4}	29
AA-tRNA	3×10^{-3}	20
Control A	1/5 of reaction	4
Control B	volume	5

Each 0.1 ml of the reaction mixture contained 0.42 mg labelled ribosomes (100 cpm) and the same components as mentioned in Table IX. After 10 minutes at 37° C, reaction was stopped by addition of 3 ml of cold 0.1% trichloroacetic acid. Tubes were allowed to stand in ice for 10 minutes, filtered on glass fiber filter (FG/C) and washed three times with 3 ml portions of cold 0.1% TCA. Filters were dried at 100° for 30 minutes and radioactivity counted under 10 ml of toluene scintillation fluid.

A = Reaction mixture for T_1-RNase digestion minus aminoacyl-tRNA

B = Reaction mixture for pancreatic RNase digestion minus animoacyl-tRNA

* = Concentration was calculated as in Table XII

** = % release was calculated from the loss of TCA precipitable counts on the filter versus the reaction with no additions.

percentage of polyphenylalanyl-tRNA complexes reacting, since polyphenylalanine chains are probably of heterogeneous lengths, and different chain lengths react at different rates. This criticism applies, in general, to all methods in which the release of nascent protein is studied. However, "percentage release" provides a measure of the relative extent of reaction and is adequate for this purpose.

7. Activity of Ado(Tyr), ACCA(Tyr) and AAUCCUUCCCCCACCACCA(Tyr)

The experimental results described in the previous section clearly demonstrate that in confirmation of the previous report (Takanami, 1964) aminoacyl-oligonucleotides and aminoacyl-adenosine can act as peptide acceptors on the ribosomes and that aminoacyl-adenosine is a weaker acceptor than aminoacyl-oligonucleotides. In order to analyze this effect in more detail, and in view of the variation in peptidyl transferase activity dependent on the nature of the amino acid side chain (Rychlik et al., 1970), this study was carried out with a specific aminoacyl-tRNA which could be used to generate various sizes of well-defined aminoacyl-oligonucleotides. Tyr-tRNA^{Tyr} was chosen for this purpose because a very long (19mer-Tyr) and a very short (4mer-Tyr)Tyrosyl-oligonucleotide, could be obtained by complete T_1-RNase digestion on E. coli Tyr-tRNA^{Tyr} and yeast Tyr-tRNA^{Tyr}, respectively. On the other hand, pancreatic RNase digestion of either of these Tyr-tRNAs would produce Ado(Tyr) (see also section 6). This would provide a reasonable means for studying the specific effect of oligonucleotide chain length on peptide bond formation. A kinetic comparison of these substrates is described below.

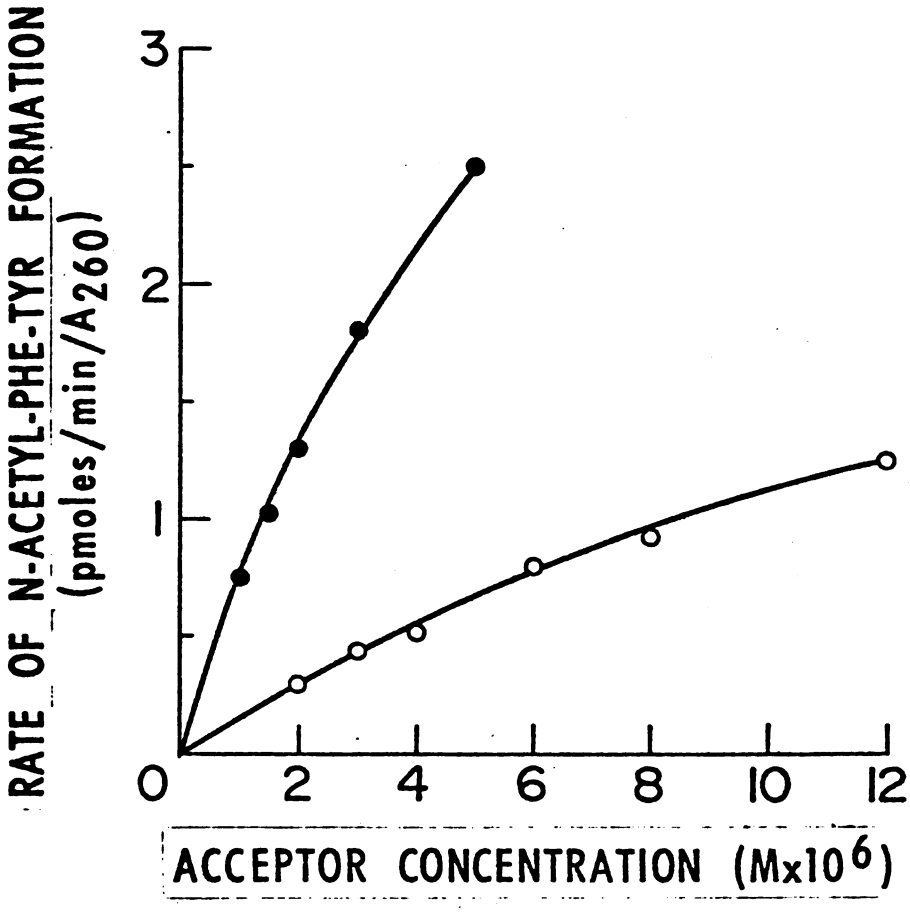
a. Kinetics of peptide bond formation using washed ribosomes.

In the first series of experiments, the activities of puromycin (a close structural analog of Ado-Tyr), ACCA-Tyr and AAUCCUUCCCCCACCACCA-Tyr (19mer-Tyr) were compared for synthesis of N-acetyl-Phe-Tyr on salt-washed ribosomes according to the procedure of Miskin et al. (1970). Figure 23 shows the rate of formation of peptide with increasing concentration of acceptor substrates in presence of N-acetyl-Phe-tRNA as

Figure 23

Rate of formation of N-acetyl-Phe-tyrosine on washed ribosomes

Each 50 µl of reaction mixture contained (prior to methanol addition) the same components as described in Chapter II.D.1.b., except that 4.0 A_{260} units of washed ribosomes and 60 pmoles of N-acetyl[^{3}H]Phe-tRNA were added and puromycin was omitted. Before the addition of acceptor, 25 µl of methanol was added and the mixture incubated for 2 minutes at 24° C. The reaction was started by the addition of [^{14}C]Tyr-oligonucleotides and incubation continued at 24° C for the time indicated below. The reaction rapidly became non-linear with time so that it was essential to test linearity with each substrate employed. The reaction was terminated by addition of 5 µl of 3 N NaOH to hydrolyze ester bonds. After incubation for 20 minutes at 37° C, 200 µl of 5 N HCl was added and the N-acetyl[^{3}H]Phe-[^{14}C]tyrosine formed was extracted from this acid solution with 3 ml of ethylacetate and counted with exclusion of [^{3}H]. The blank value for each tyrosyl-oligonucleotide concentration was obtained by omitting N-acetyl-[^{3}H]Phe-tRNA. The values obtained were corrected for the sample size and for extraction efficiency (96% as determined by independent experiment, see Chapter II.D.4). The results are expressed as pmole product formed per minute of reaction per A_{260} unit of ribosomes. ●—●, ACCA-Tyr. Incubation was for 1.5 minutes. The rate of reaction was linear up to 2.0 minutes. O—O, 19mer-Tyr. Incubation was for 2.5 minutes. The rate of reaction was linear up to 4 minutes.


RATE OF N-ACETYL-PHE-TYR FORMATION
(pmoles/min/A260)
3
2
1
0
2
4
6
8
10
12
ACCEPTOR CONCENTRATION (Mx10⁶)

donor. Apparent K_m and V_{max} were obtained from an $\frac{S}{V}$ versus S plot (Dowd and Riggs, 1965) of these data and shown in Figure 24. For comparison, the rate of peptide formation with puromycin was also determined. A kinetic plot of $\frac{S}{V}$ versus S for puromycin is shown in Figure 25. These results are summarized below (Table XIV).

It should be pointed out that the experiments described above were conducted in the presence of 33% (v/v) methanol because methanol was obligatory for peptide bond formation with oligonucleotide acceptors under these assay conditions. Preliminary experiments using ACCA(Tyr) or CACCA(Phe) showed that no reaction took place in its absence (< 0.05 pmole/A_{260} of peptide product was formed after 5 minutes at 1 μM ACCA-Tyr). Although the reaction containing methanol is unnatural, there is evidence to support the fact that the reaction mechanism is identical with peptide bond formation in protein biosynthesis (Monro et al., 1968; Monro and Vasquez, 1967; Lucas-Lenard and Lipmann, 1967). An absolute requirement for alcohol has also been observed for the "fragment reaction" at the P site for formylmethionyl-puromycin formation from formyl-methionyl-hexanucleotide and puromycin (Monro and Marcker, 1967).

In contrast to the absolute requirement for alcohol for activity with aminoacyl-oligonucleotides, puromycin reacted readily in its absence. Thus, to delineate the effect of methanol, the reactivity of puromycin was studied both in the absence and presence of methanol. Under identical conditions to those described in Figure 23 but with methanol omitted, the K_m for puromycin as shown in Figure 25, remained the same 122 μM vs. 149 μM) and the V_{max} decreased only 2.5 fold (2.6 pmole/min A_{260} vs. 6.9).

To investigate the reason for the higher K_m of 19mer-Tyr versus 4mer-Tyr, the binding of these fragments to 70S ribosomes was studied.

Figure 24

Determination of K_m for the formation of N-acetyl-Phe-Tyrosine for N-acetyl-Phe-tRNA and 19mer-Tyr and ACCA-Tyrosine on washed ribosomes

S/V versus S plot was drawn from the data of Figure 24. K_m and V_{max} were calculated as described in Chapter II.D.5.

○ , 19mer-Tyr, ● , 4mer-Tyr.

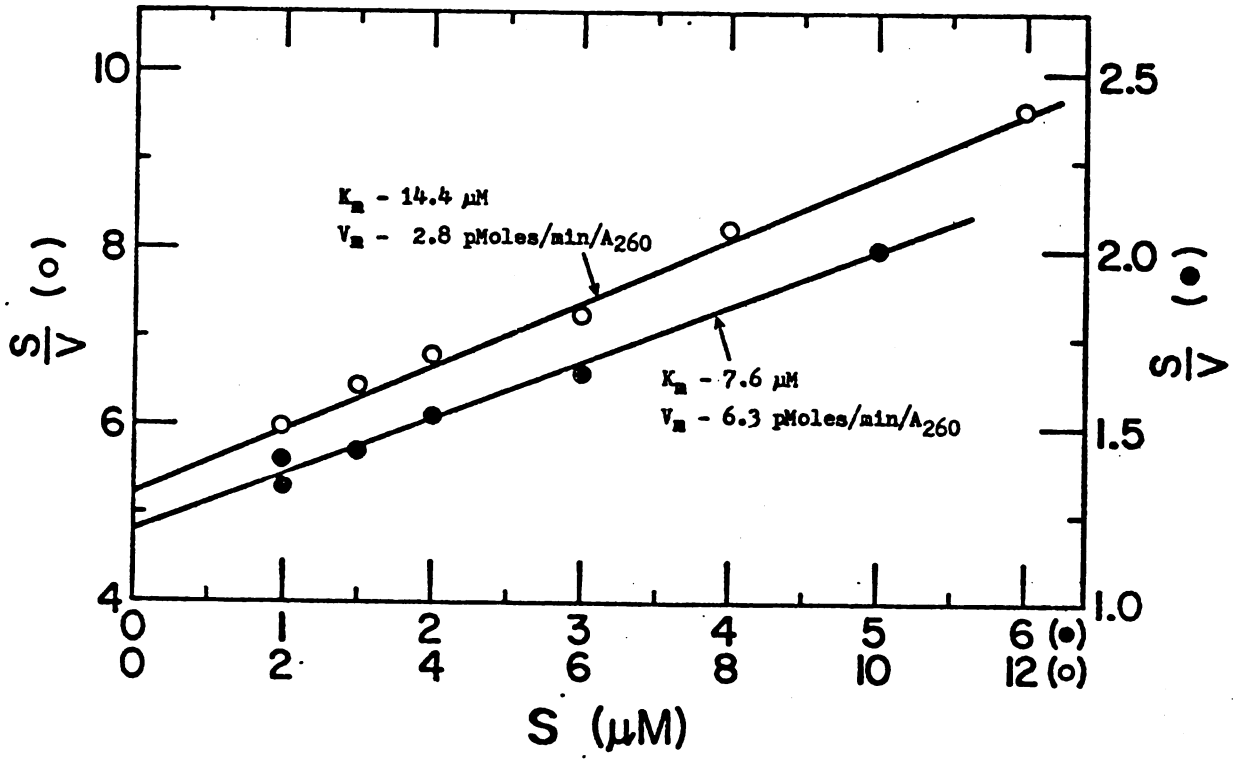

10
8
6
4
2.5
2.0
1.5
1.0
S|> (○)
S|> (●)
K_m - 14.4 μM
V_m - 2.8 pMoles/min/A_{260}
K_m - 7.6 μM
V_m - 6.3 pMoles/min/A_{260}
0 1 2 3 4 5 6 (●)
0 2 4 6 8 10 12 (○)
S (μM)

Figure 25

Rate of formation of N-acetyl-Phe-puromycin from N-acetyl-Phe-tRNA and puromycin on washed ribosomes in presence and in absence of methanol

Components and the conditions of the reaction are as in Figure 24 except that reactions were started by the addition of puromycin at the indicated concentrations. Incubations were for 1.5 minutes; the reaction was linear up to 2 minutes. Reactions were terminated and processed as described in Chapter II.D.1.a. The radioactivity of a blank incubation in absence of puromycin was substracted and the results are expressed as in Figure 24. An S/V versus S plot was constructed from these data. K_m and V_{max} were calculated as described in Chapter II.D.5.

● , minus methanol

○ , plus methanol

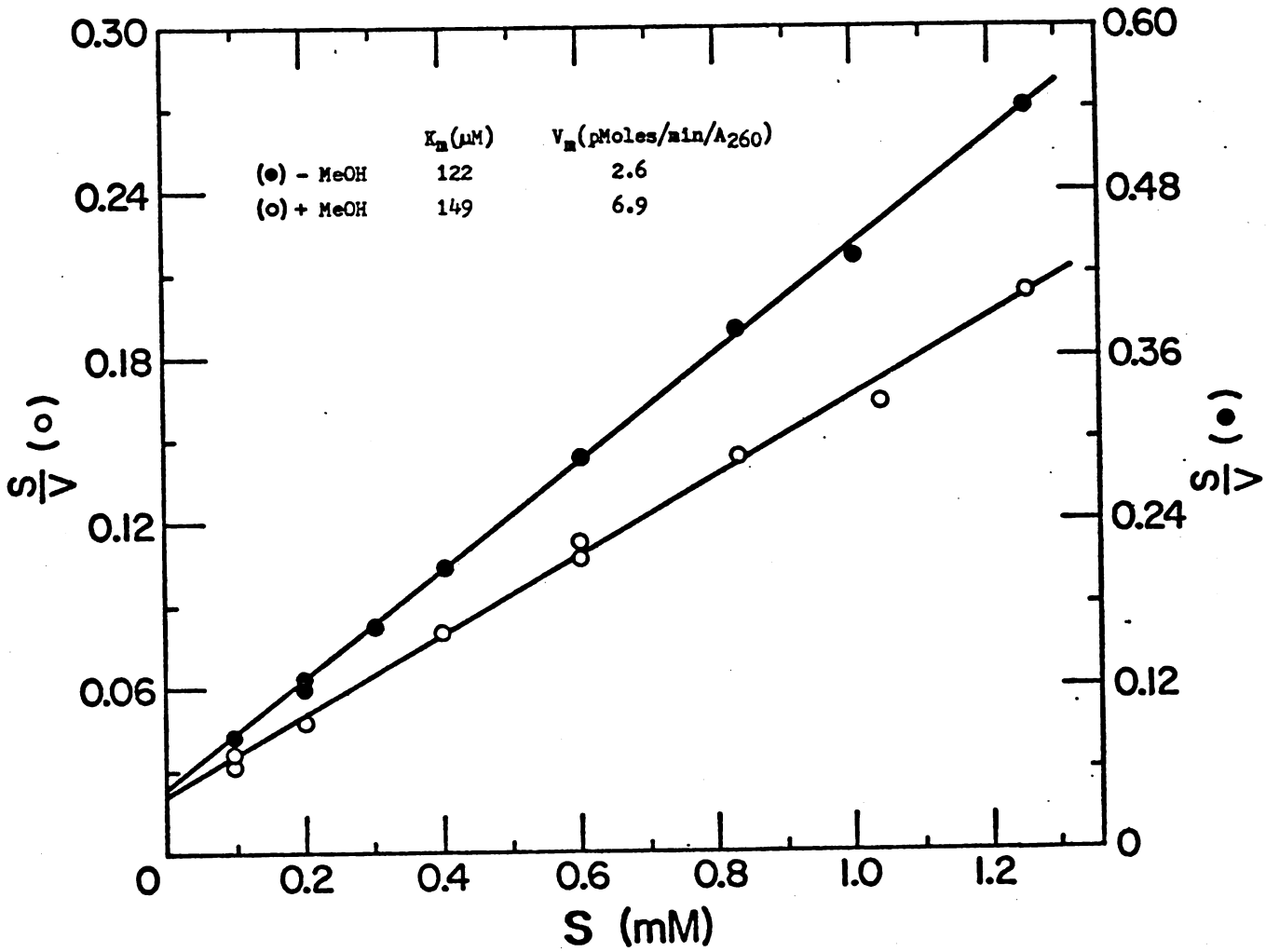

0.30
0.24
0.18
0.12
0.06
0
0.60
0.48
0.36
0.24
0.12
0
0.2
0.4
0.6
0.8
1.0
1.2
S (mM)
S/V (○)
S/V (●)
Km(μM)
Vm(pMoles/min/A260)
(●) - MeOH
122
2.6
(○) + MeOH
149
6.9

TABLE XIV

Kinetic Constants for Various Acceptors at the Peptidyl Transferase Center of Ribosomes and Polysomes

Acceptor Substrate	Ribosomes		Polysomes	
	K_m	V_{max}	K_m	V_{max}
	μM	pmole/min/A_{260}	μM	pmole/min/A_{260}
Puromycin	149(122)	6.9(2.6)	3.0	5.5
A-Tyr	--	--	4.9	7.3
ACCA-Tyr	7.6	6.3	6.5	14.2
AAUCCUUCCCCC-ACCACCA-Tyr	14.4	2.8	> 400	> 10

Kinetic constants were obtained from the S/V versus S plots of the data shown in Figures 24, 25, and 28. Reaction mixtures contained 33% methanol as described in Methods and Figures. Value in ( ) was obtained in absence of methanol.

Alcohol has been shown to stimulate the extent of binding of CACCA(Phe) to purified 70S ribosomes and it has been suggested (Pestka et al., 1970) that this binding even though observed under conditions of high salt and 20% alcohol, nevertheless reflects specific binding to ribosomal functional sites. The extent of binding of ACCA(Tyr) and 19mer-Tyr to washed 70S ribosomes in the presence of 20% (v/v) ethanol is shown in Figure 26. The binding was proportional to Tyr-oligonucleotide concentration up to the highest level tested. Approximately 45% of the added tetramer-Tyr and 21% of the 19mer-Tyr were bound to ribosomes. In the absence of alcohol no binding took place with either fragment. It is interesting to note that the ratio of the binding efficiency of these fragments coincides with the ratio of their K_m values.

It is clear from the data summarized in Table XIV that the effect of adding 3 nucleotides to A-Tyr (assumed to be simulated by puromycin in view of the close structural analogy - see Figure 20C) resulted in a 19-fold reduction in K_m but no change in V_{max}. Further increase in chain length to 19 increased the K_m two times and decreased the V_{max} two-fold. This effect was much less than expected, possibly due to the unphysiological conditions of the assay, namely 33% methanol. Since aminoacyl-oligonucleotides required alcohol to be active in this system, it was necessary to turn to a system which did not require alcohol and high salt.

b. Kinetics of peptide bond formation using washed polysomes

Because of apparent disparate results of inhibitors on peptide bond synthesis in model systems and intact cells, Pestka (1971) recently developed a polyribosome system for the study of peptide bond formation

Figure 26

Binding of Tyrosyl-oligonucleotides to ribosomes as a Function of Tyrosyl-oligonucleotide concentration in the presence and absence of ethanol.

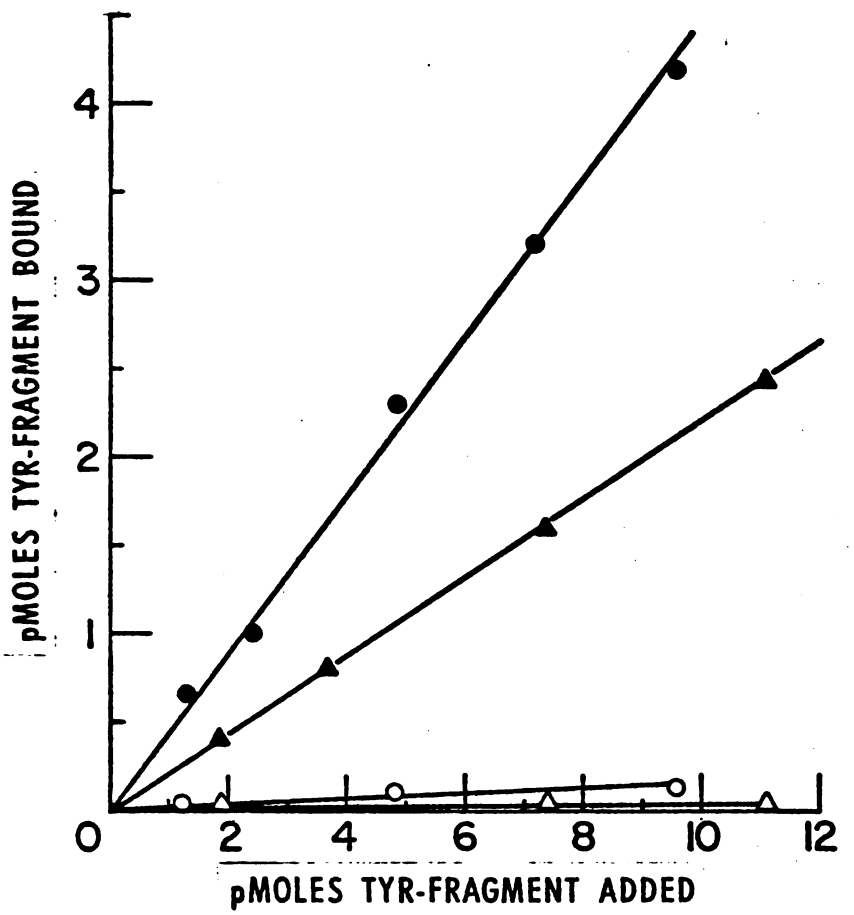
Binding of Tyrosyl-oligonucleotides to ribosomes were carried out according to the method described by Pestka et al. (1970). Each reaction mixture contained in 50 µl, 50 mM Tris-acetata, pH 7.2, 400 mM potassium acetate, 60 mM ammonium chloride, 40 mM magnesium acetate, 20% (v/v) ethanol, 3.3 A_{260} units of washed ribosomes and Tyr[^{14}C]oligonucleotides as specified. Tyrosyl-oligonucleotides were added last to start the reaction. Reactions were incubated for 30 minutes at 24° C and terminated by the addition of 3 ml of cold buffer containing 50 mM Tris-acetate, pH 7.2, 400 mM potassium chloride, 40 m M magnesium chloride and 20% (v/v) ethanol and immediately passed through a Millipore filter to absorb the ribosomes (Nirenberg and Leder, 1964). The tubes and the filters were then washed an additional three times with 3 ml portions of the dilution buffer mentioned above. The filters were dissolved in 10 ml of Bray's (Bray, 1960) solution and counted.

△ ,19mer-Tyr

○ ,4mer-Tyr

open symbols, minus ethanol

closed symbols, plus ethanol


pMOLES TYR-FRAGMENT BOUND
0
1
2
3
4
0
2
4
6
8
10
12
pMOLES TYR-FRAGMENT ADDED

(Pestka, 1972) in which polyribosomes isolated from E. coli are used as an enzyme-substrate complex containing peptidyl-tRNA. Peptidyl-puromycin synthesis on these polyribosomes has distinctly different characteristics and requirements from those of peptidyl-puromycin synthesis with synthetic donors such as N-acetyl-Phenylalanyl-tRNA or formylmethionyl-tRNA and washed ribosomes and is thought to more closely resemble the in vivo situation (Pestka, 1972).

Washed polysome preparations (RNase free) were employed in these studies in order to preserve the integrity of the oligonucleotide moiety. Before use polyribosomes were checked for nuclease activity. This was done by incubation of TCA precipitable aminoacyl-oligonucleotides (e.g. 19mer-[^{14}C]Tyr (Chapter II.3.a.) and 13mer-[^{14}C]Val (Krauskopf et al., 1972) with the polysome preparation for 10 minutes at 37^{o} and measurement of the TCA precipitable radioactivity remaining. 100% recovery was considered to mean that the preparation was free from nuclease contamination. The effect of varying acceptor concentration and chain length on peptide bond formation in this system is illustrated in Figure 27. As seen in this figure, the formation of peptidyl-tyrosine follows simple enzyme kinetics with respect to acceptor substrates which gives linear S/V versus S plots as shown in Figure 28. The relevant kinetic constants are summarized in Table XIV. In contrast to the purified ribosome system, puromycin, A-Tyr, and ACCA-Tyr all have similar K_m and V_{max} values. Note that the K_m for ACCA-Tyr is the same in both the polysome and ribosome systems. It appears that puromycin has (and presumably A-Tyr) anomalously high K_m values when salt-washed ribosomes are used. The greatest difference in response was seen with 19mer-Tyr, the K_m for this oligomer being about 100 times greater than that for the

Figure 27

Rate of formation of peptidyl-tyrosine and peptidyl-puromycin on polyribosomes

Reaction components, conditions and assay procedure are described in Chapter II.D.2.a. and b. Incubations were at 24° for the times indicated below.

▲ , puromycin, incubated for 45 seconds; the rate of reaction was linear upto 60 seconds.

○ , A-(Tyr), incubated for 45 seconds; the rate of reaction was linear upto 60 seconds.

△ , ACCA-(Tyr), incubated for 30 seconds; the rate of reaction was linear upto 45 seconds.

● , 19mer-Tyr, incubated for 5 minutes; the rate of reaction was linear upto 10 minutes.

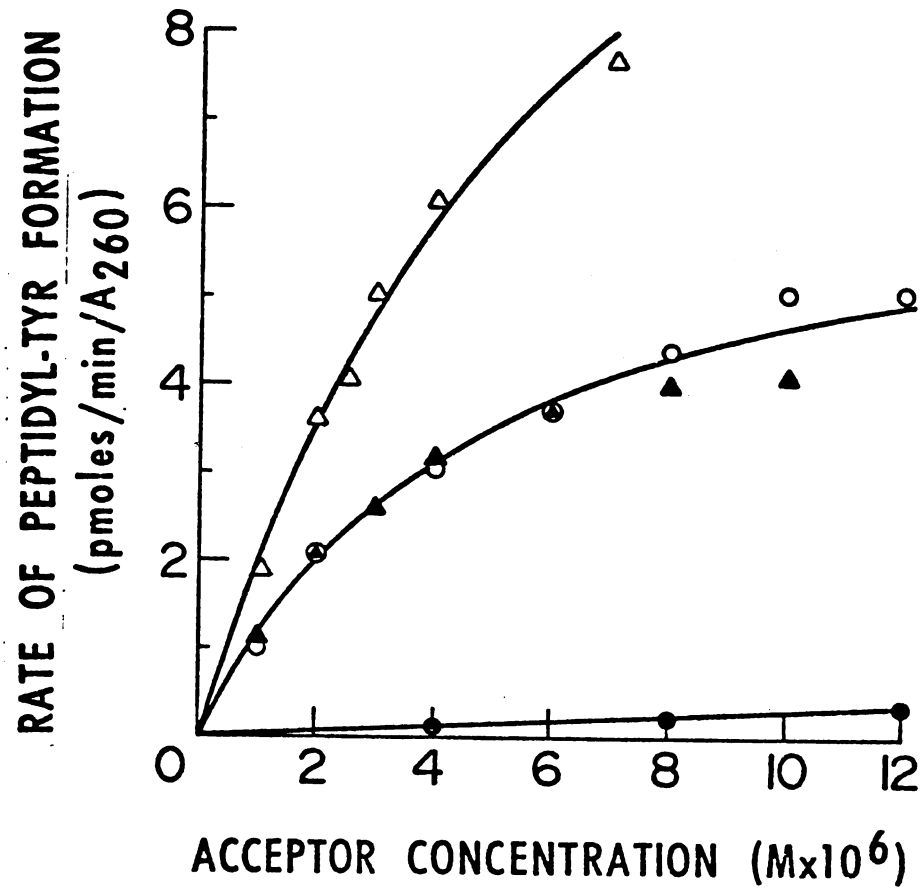
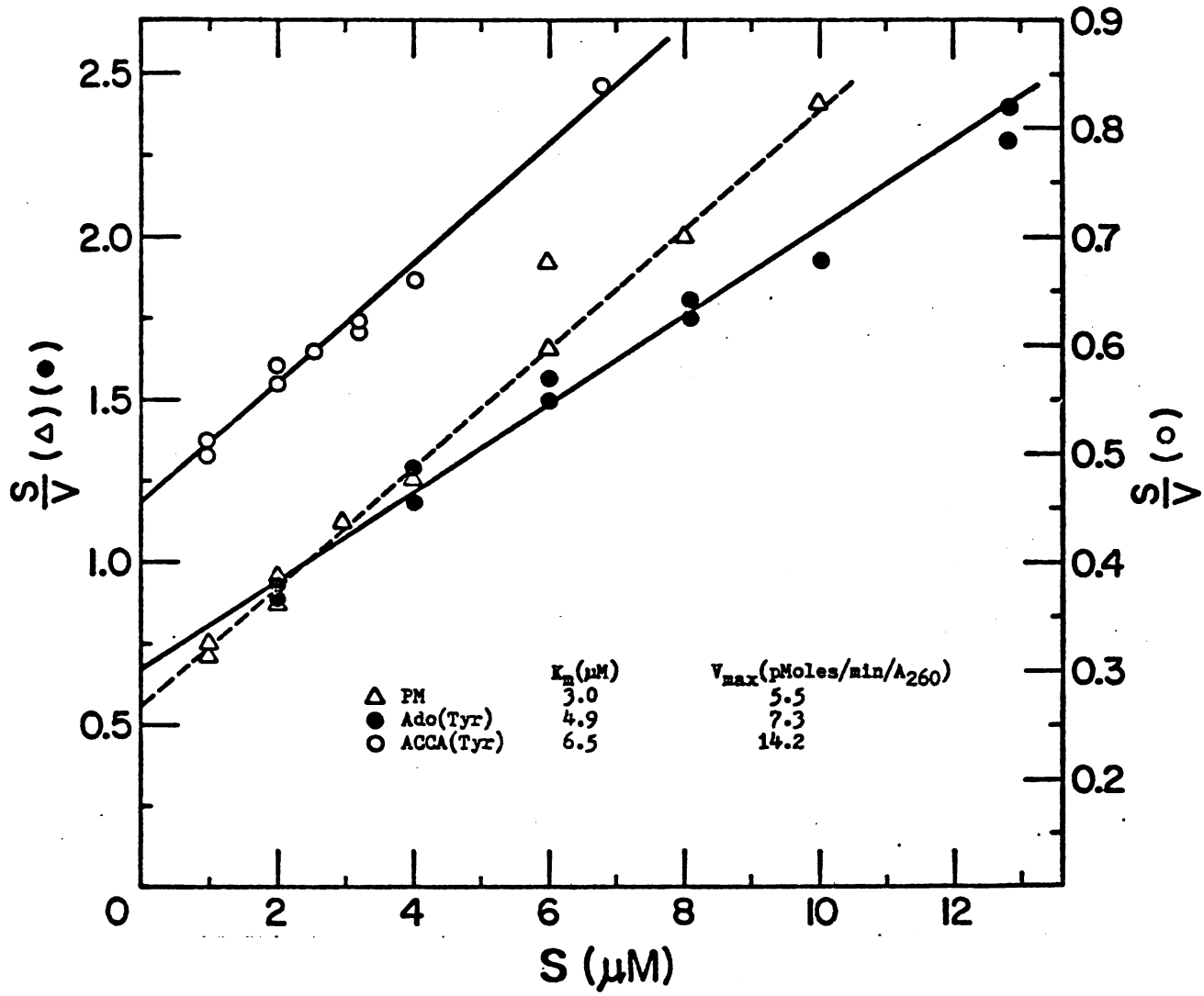

RATE OF PEPTIDYL-TYR FORMATION
(pmoles/min/A260)
8
6
4
2
0
2
4
6
8
10
12
ACCEPTOR CONCENTRATION (Mx10⁶)

Figure 28

Determination of K_m for the formation of peptide product with 19mer-Tyr, 4mer-Tyr, Ado(Tyr) and puromycin on polyribosomes.

Data from figure 28 are drawn as S/V versus S plot. K_m and V_{max} were calculated as described in Chapter II.D.5.

Δ, Puromycin; ●, Ado(Tyr); O, ACCA(Tyr).


S/V (△)(●)
S/V (○)
S (μM)
0
2
4
6
8
10
12
0.5
1.0
1.5
2.0
2.5
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
△ PM
● Ado(Tyr)
○ ACCA(Tyr)
Km(μM)
3.0
4.9
6.5
Vmax(pMoles/min/A260)
5.5
7.3
14.2

other oligomers in the polysome system, and some 30 fold higher than that measured when 19mer-Tyr was tested in the ribosome system.

The true kinetic constants for the 19mer-Tyr could only be estimated since the rates were so low and more material was not available. The data of Figure 27 (shown in expanded form in Figure 29) show that the rate of reaction with 19mer-Tyr is still increasing linearly at the highest concentration of 19mer-Tyr employed. This means that the velocity is so far below the K_m that ordinary means of analysis to determine the kinetic constants cannot be applied. However, these data may still be used to obtain an approximate limit on K_m and V_{max}. Two approaches were used.

(i) (Mertes et al., 1972) Since in the region of the linear response $V = (V_{max}/K_m)$[19mer-Tyr], from the slope of the graph, (Figure 29) $V_{max}/K_m = 2.7 \times 10^4$ (pmoles/min/A_{260} M^{-1}). The response (V) will depart from linearity at a [19mer-Tyr] concentration at which [19mer-Tyr] $\simeq$ (f)(K_m) where f is the smallest fractional deviation from linearity which could be detected. It is estimated that the velocity at the highest concentration point (12 μM) is within 3% of linearity so that $K_m \gg [12 \times 10^{-6}\ M]/0.03 = 400\ \mu M$. Since $V_{max} = 2.7 \times 10^4\ K_m$, $V_{max} \lesssim$ 10.8 pmoles/min/A_{260}. The values represent approximate lower limits for K_m and V_{max}.

(ii) An approximate value of K_m and V_{max} was also obtained from the double reciprocal plot (not shown) of the data from Figure 29. The best line intercepting the abscissa beyond the ordinate gave a value of approximately 500 μM for K_m and 10 pmoles/min/A_{260} for V_{max}. These values are taken to be the most likely ones since they fall in the same range of values calculated by (a).

(c) Effect of complex formation with oligoinnosinate on the reactivity of 19mer-Tyr for peptide bond formation

The sequence of 19mer-Tyr contains a preponderance of C residues in the region normally forming H-bonds in the tRNA cloverleaf structure. Nine of the 12 residues normally base-paired are C, 2 are U, and one is A. Consequently, one may mimic the double-strandedness of this part of the tRNA cloverleaf by complexing 19mer-Tyr with poly I. Use of I instead of G has the double advantage that the aggregation tendency of oligo G is minimized and by "Wobble" base pairing (Crick) even the single A residue may be incorporated into a double stranded structure. "Wobble" also allows G-U or I-U base pairs, so that complete pairing is possible.

The effect of complexing with $(I_p)_5I$ on the rate of reaction of 19mer-Tyr is shown in Figure 29. There was no detectable effect either in stimulating or further reducing the already low level of activity of 19mer-Tyr even when a 10-fold excess of oligo I was used. This means either that complex formation had no effect, or that no (< 5%) complex formation occurred. 19mer-Tyr did in fact complex with $(I_p)_5I$ under the reaction conditions, as shown by the experiment in Table XV, which clearly demonstrates an effect of $(I_p)_5I$ addition in the case of 19mer(Tyr). The rationale for the experiment is as follows: 19mer(Tyr) normally reacts at a very slow rate compared to ACCA(Tyr) and Ado(Tyr) (Figure 27). In the presence of nuclease, however, such as exists as a contaminant in the unwashed polyribosomes used in this experiment, the 19mer(Tyr) is rapidly hydrolyzed to small fragments that react well. In the absence of $(I_p)_5I$, all 3 fragments gave similar amounts of product formed from 19mer-Tyr dropped to 50% of that found in the absence of $(I_p)_5I$. Moreover, the

Figure 29

Effect of $(I_p)_5I$ on the rate of peptide bond formation between 19mer-tyrosine and peptidyl-tRNA on polyribosomes.

19mer-tyrosine, 0.2, 0.4, and 0.6 nmoles, corresponding to 0.08, 0.16, and 0.24 A_{260} units was incubated in the complete polysome assay system as described in Figure 28 but with polysomes omitted for 2 minutes at 24°C in the absence (○) and presence (●) of 0.8, 1.6, and 2.4 A_{260} units, respectively of $(I_p)_5I$. One A_{260} unit of polysomes was then added and incubation continued for an additional 5 minutes at 24° C. The reaction was stopped and assayed as described in Figure 28.

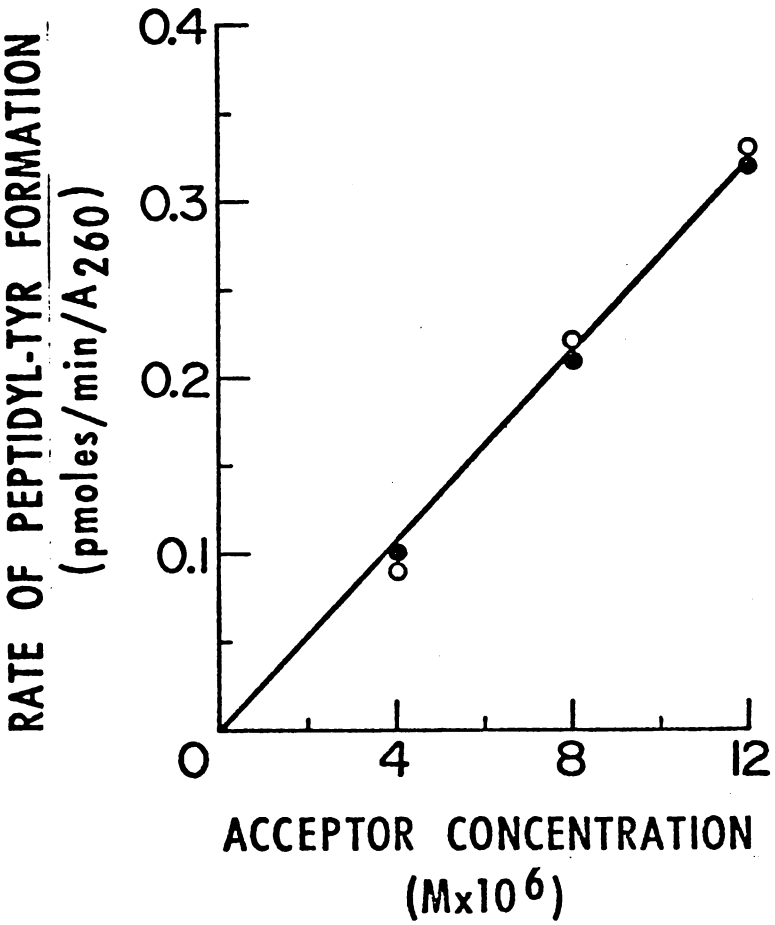

RATE OF PEPTIDYL-TYR FORMATION
(pmoles/min/A260)
0.4
0.3
0.2
0.1
0
4
8
12
ACCEPTOR CONCENTRATION
(Mx10⁶)

TABLE XV

Formation of Complex between 19mer-Tyr and oligoinosinate

	% of Maximum Peptidyl-tyrosine formed		
$(I_p)_5I$ (A_{260} unit)	19mer-Tyr	ACCA-Tyr	A-Tyr
	100 (3.4)	100 (2.4)	100 (3.3)
.02	71	-	-
.04	58	-	100
.1	55	96	97
.2	50	100	94
.4	51	100	98

200, 150 and 250 pmoles of 19mer-Tyr, ACCA(Tyr) and Ado(Tyr) respectively were incubated in a complete polysome assay system as described in Figure 27 but with polysomes omitted for 2 minutes at 24° C in the presence of the indicated amounts of $(I_p)_5I$. 2.8 A_{260} units of unwashed polysomes was then added and the mixture incubated for 8 minutes at 37° C. The reaction was stopped and processed as described in Figure 27. The values in parentheses are the pmoles of peptidyl-tyrosine formed.

amount of $(I_p)_5I$ needed to generate the maximal effect, ca 0.04 A_{260} units is about what would be expected to titrate 200 pmoles of 19mer-Tyr added (equivalent to 0.08 A_{260} units). The failure to decrease the reaction to ca 5% as expected presumably is due to the failure of the double-stranded complex to completely protect the 19mer from the amount of nuclease added. In any case, it is clear that at least 50% complexing took place, which had no effect on the peptidyltyrosine formation.

It was assumed that double-stranded formation of 19mer-Tyr with $(I_p)_5I$ would protect 19mer-Tyr from hydrolysis by contaminating nucleases in the unwashed polysomes.

Independent evidence for the complexing of 19mer-Tyr with complementary oligomers has also been obtained using oligo G in a similar incubation system (Beltchev and Grunberg-Manago, 1970). In this case, formation of the complex generated a competitive inhibitor of the rate of aminoacylation of $tRNA^{Tyr}$ showing that the double-stranded fragment can function in a different way from the single stranded 19mer in some instances. In the present example, however, formation of a structure analogous to the amino acid stem of the tRNA cloverleaf did not affect the activity of 19mer-Tyr with polyribosomes.

CHAPTER IV

DISCUSSION

A. Oxidized-reduced tRNA.

The hydroxyl groups of the 3'-terminal ribose of tRNA are directly involved in various steps of protein biosynthesis, e.g., aminoacylation, T-factor recognition and peptide bond formation (see review of Lucas-Lenard and Lipmann, 1971). Aminoacyl and peptidyl residues are bound by as ester linkage to the 2'-or 3'-hydroxyl group of the ribose residue of the terminal adenosine and exchange rapidly between these two cis-diol positions (Wofendon et al., 1964; Griffin et al., 1966; McLaughlin and Ingram, 1965). This transacylation might also occur enzymatically during the various steps of protein biosynthesis as an essential functional element. A part of the work described in this thesis relates to the activity of tRNA in protein synthesis from this stand point. Chemically oxidized-reduced $tRNA^{Phe}$ was selected for these studies.

The oxidation of cis-glycols by sodium periodate is a well known reaction in carbohydrate chemistry (Schmidt, G., 1968), and has been used repeatedly in the tRNA field ever since Priess et al., (1959) and Hecht et al., (1959) showed that the amino acid attachment site in tRNA could be destroyed by periodate treatment. Subsequent reduction of the dialdehyde to the dialcohol has also been used as a means of radioactively labeling the 3'-end of tRNA (RajBhandary, 1968; Leppla et al., 1968). Several reports prior to this work described the ability of yeast $tRNA^{Phe}$ to be enzymatically acylated following oxidation-reduction (Chen and Ofengand, 1970; Ofengand and Chen, 1971; Tal et al., 1972). Indeed it was the possibility of being able to acylate such a modified tRNA which provided the impetus for this series of studies.

The findings reported here show clearly that cleavage of the C2'-C3' bond of the 3'-terminal ribose renders the aminoacyl-tRNA (or fragments derived from it) totally inactive as either a donor or acceptor at the peptidyl transferase catalytic center of the ribosome. Several hypotheses could be advanced as explanation for this effect. For example, the ribose structure is destroyed by this cleavage and it has been shown (Haar, v.d., et al., 1972) that the original planar structure becomes distorted by rotation about the C_1'-O-C_4' acetal linkage. The adenine base probably also occupies a different spatial position with respect to the rest of the structure, and other effects could no doubt be proposed. However, the fact that chemically synthesized Ado^{ox-red}(Phe) was active, rules out all such hypotheses which are consequential to opening of the ribose ring. It is therefore proposed that the sole difference, and explanation, for the contradictory results obtained with the chemically synthesized and biosynthetic Ado^{ox-red}(Phe), lies in the fact that the chemically synthesized product is almost certainly a mixture of the 3' and 2' isomers, while the biosynthetic product is probably a single isomer. It is also deduced that cleavage of the C3'-C2' bond eliminates the acyl migration of the phenylalanyl residue between the 3'- and 2'- hydroxyls which is known to be extremely rapid in aminoacyl adenosines and similar compounds (Griffin et al., 1966). This point follows automatically since the biosynthetic product was completely inactive and never showed any sign of a slow conversion to an active form even when used at very high concentrations. In view of the fact that puromycin, a 3'-derivatives, is a functional acceptor (Nathans and Neidle, 1963) it is logical to assume that the same is true for the Ado^{ox-red}(Phe) compounds. Therefore, it follows

that (a) the product synthesized by the Phe-tRNA synthetase must be in 2'-isomer of Phe-tRNA^{ox-red} and (b) that peptidyl transferase is specific for the 3'-isomer as an acceptor. The location of the phenylalanyl residue on Ado^{ox-red}(Phe), derived from this tRNA has recently been verified by direct methods. The proton magnetic resonance spectrum of carbobenzoxyphenylalanyl-adenosine^{ox-red} from PhetRNA^{ox-red} shows unequivocally that the product is the 2'-isomer, and TLC analysis by the method of Chladek et al., (1973) showed that more than 98% of the sample was 2'-isomer (Ofengand, J., personal communication).

The idea that only the 3'-isomer is the one functional acceptor as a peptidyl transferase in the synthesized 2' and 3' Ado^{ox-red}(Phe) mixture, has been supported by recent work of Chladek et al., (1973).

Binding of the donor and acceptor molecules to their proper ribosomal sites is an essential feature of the peptide bond formation (Lucas-Lenard and Lipmann, 1971). It was therefore important to study how the modified Phe-tRNA^{ox-red} behaved in such binding experiments. Under the same conditions used for puromycin reaction (20 mM Mg^{++}), no difference in N-Ac-Phe-tRNA^{ox-red} binding to the P site was observed. Affinity labeling experiment described in Chapter III.2.c lends a strong support to this observation. It demonstrated clearly that BrAc-Phe-tRNA^{ox-red} reacts covalently with the same 50S protein (believed to be located near the P-site) and with similar efficiency as the control tRNA. Nevertheless, the modified tRNA molecule cannot take part in peptide bond formation since N-Ac-Phe-tRNA^{ox-red} and the fragment CACCA^{ox-red}(N-Ac-Phe) both were completely unable to donate their peptidyl moieties to puromycin, suggesting that peptidyl transferase may also be 3'-specific at the P-site.

If the above reasoning is correct it follows that initiation complex formation should likewise be specific for the 2'-ester of aminoacyl-tRNA. However, the possibility in this case that the lack of activity is caused by structural alterations correlated with the opening of the ribose ring, cannot be completely ruled out. Most likely the specificity is imparted by the initiation factors rather than by the ribosomal binding site since it has been shown in this work that Br-Ac-Phe-$tRNA^{ox-red}$ (2'-isomer) binds to the protein L_2 located near the P site as efficiently as the Br-Ac-Phe-$tRNA^{cont}$. Generally the P site is considered to be equivalent to the initiator site on 70S ribosome. Similar results have previously been reported by Ofengand and Chen (1971) using Phe-$tRNA^{ox-red}$.

On the basis of this work, one is tempted to predict further that the esterification of the 3'-hydroxyl of $tRNA^{ox-red}$ is not an abberation of the Phe-tRNA synthetase due to the structural modification of its substrate tRNA, but that this position is the primary site of esterification for yeast-Phe-tRNA synthetase. Recent observations suggest (Ofengand, J., personal communication) that this is generally true for all synthetases. The 2'-hydroxyl is the more reactive one from a chemical standpoint (Zamecnik, P., 1962). An unequivocal evidence to this effect is recently provided by Sprinzl and Cramer (1973) who showed that only the tRNA with 3'-deoxyadenosine at the 3'-terminal end is aminoacylated but not the tRNA ending in 2'-deoxyadenosine. Furthermore, the K_m for 3'-deoxyadenosine-tRNA was the same as that of the normal and 2'-deoxyadenosine-tRNA was a competitive inhibitor with $K_i = K_m$ in this reaction (Cramer, F., et al., 1968). All these suggest that there is no difference in recognition for the open ring structure by the synthe-

tase.

In the light of the above discussion and the results presented in this work there can be no doubt that the 2'-isomer of Ado^{ox-red}(Phe) and of $CACCA^{ox-red}$(Phe) are completely inactive as acceptors in the peptidyl transferase reaction. However, as shown in Figure 15, Phe-$tRNA^{ox-red}$ (the amount of Phe-$tRNA^{cont}$ in this preparation was estimated to be < 1%). see Figure 5 showed a little activity (12% of control) as an acceptor. One can raise a possibility that a small population of the 2'-isomer of Phe-$tRNA^{ox-red}$ may possess a correct stereochemical orientation of the 2'-amino group afforded by a structural stabilization of the bound tRNA molecule due to the interactions between several segments of tRNA and ribosome. Such stabilizing interactions would be absent in 2'-isomers of simple acceptors like puromycin, Ado^{ox-red}(Phe) and $CACCA^{ox-red}$(Phe), hence their inactivity. The fact that the 3'-isomers of the above simple acceptors are active, suggests that the 3'-isomer of aminoacyl-tRNA (or Phe-$tRNA^{ox-red}$) reacts with the donor molecule readily by virtue of an effective binding to the peptidyl transferase center.

Fraser and Rich (1973) synthesized phenylalanyl-3'-amino-3'-deoxy-tRNA and observed that in a poly U-directed protein-synthesizing system, the molecule was capable of receiving an N-acetyl-Phenylalanyl moiety from the donor site of the ribosome. However, the ribosome was unable to cleave the amide bond connecting phenylalanine to the tRNA molecule; hence the phenylalanyl-3'-amino-3'-deoxy-tRNA has acceptor but not donor activity in protein synthesis. It should be pointed out that the 3', 2' acyl migration was not possible in the molecule. Recently, Ringer and Chaldek (1974a) reported the activity of "non-isomerizable" CA(2'-Phe) 3'-H, CA(2'-Phe)3'-Me, CA(3'-Phe)2'H and CA(3'-Phe)2'-Me. Their result

showed that although only 3'-O-phenylalanyl derivatives acted as acceptors in peptide transfer, both the 2'- and 3'-O-phenylalanyl derivatives were potent inhibitors of peptidyl transferase, indicating that the 3'-terminus of 2'-O-aminoacyl-tRNA can be recognized by the peptidyl transferase A-site. Normally, at physiological pH, acyl migration would almost instantaneously produce a mixture of 3' and 2' isomers even if the original biosynthetic process were 2'-specific, so that specificity for the 3'-isomer by the other enzymes involved in protein synthesis would not be a problem. It therefore appears that it is not a single isomer of aminoacyl-tRNA that is employed throughout the various stages of protein biosynthesis.

B. Tyrosyl-oligonucleotides:

It was envisioned that some aspect of the structure of aminoacyl-tRNA compared to small aminoacyl-oligonucleotides (including aminoacyl-adenosines and puromycin) prevented access of the aminoacyl-adenosine end of tRNA to the peptidyl transferase catalytic center. This idea was tested by comparing the reactivity of tyrosyl-oligonucleotides of different chain lengths as acceptor substrates. Since no codon-mediated interaction is expected with these modified substrates, the reactivity is a direct reflection of the function of chain length to the ease of their accessibility to enzymic catalytic center. This is not possible by using whole aminoacyl-tRNA since interactions at locations other than those involving peptidyl transferase complicate the situation.

For this purpose tyrosyl-oligomers were chosen because of the following reasons: (a) Peptidyl transferase has been known to show widely varying affinities for acceptors depending on the amino acid

portion of the molecule and aromatic amino acids have the highest affinity; their interaction with postulated hydrophobic locus at the A site is enhanced by the pi electron system of the aromatic ring (Rychlik et al., 1970). Also tyrosine and purine rings can form alternating stacks (Sundaralingam and Arora, 1969) which is considered as an active form of the molecule sought after by the A subsite, and (b) mono, tetra and 19mer could conveniently be obtained from Tyr-tRNA of yeast and E. coli by appropriate enzymatic treatments.

In this report reactivity of the aforesaid tyrosyl-oligomers in the peptide transfer reaction is presented in terms of the kinetic parameters. The effect of chain lengths on acceptor K_m is evaluated both in a purified ribosome system and a more physiological polysome preparation. This constitutes the first effort of the kind in assessing the specificity of ribosomal peptidyl transferase. The K_m values for puromycin observed in this work (202 μM using the purified ribosome assay) agrees with previously published values of 180 μM (Fahenstock et al., 1970) and 300 μM (Fico and Coutsogeorgopoulos, 1972) obtained under similar conditions. The value observed in the polysome system (3.0 μM) also agrees with the literature value of 2.4 μM reported earlier by Pestka (1972) for unwashed polysomes. However, comparison of the data in Table XIV for tyrosol-oligomers as well as for puromycin makes it clear that the value obtained for puromycin with polysomes is not unusually low but rather that the purified ribosome value is anomalously high. At the present, the reason for this is not understood.

Comparison of the K_m values for 4mer and 19mer in the two assay systems shows clearly that the more "physiological system" can reveal differences obscurred by the ribosome assay. This is readily seen by

comparing the 19mer K_m value in the two systems. Moreover, the fact that the tetramer K_m value is the same in both systems suggests that the discriminatory ability of the polysome preparation is abolished by the time the oligonucleotide chain length is reduced to 4 units. It should be emphasized at this point that both the polysome and the ribosome preparations were free from detectable ribonuclease activity so that the observed apparent K_m are true for the oligomers indicated. The pattern of their reactivity with the salt-washed ribosomes also parallels their binding abilities. 19mer binds half as much as the tetramer under identical conditions.

The differential reactivity observed in these studies for different sized oligomers suggested that peptidyl transferase may indeed be shielded by the structure of the polyribosome from attack by the bulky aminoacyl-tRNA in the cytoplasm. On the other hand, acceptors like puromycin, aminoacyl-adenosines and relatively smaller aminoacyl-oligomers are accessible due to their smaller size, to the ezymic center and therefore able to react. This situation however, is more effective for polysome structure (which better mimics the true physiological system) than the salt-washed "open" and distorted ribosomes. In order for peptide bond formation to occur, the enzyme site must be made available to the aminoacyl acceptor end of the incoming codon-specified aminoacyl-tRNA. This may occur either simply by virtue of specific complexing to the ribosome at the A site or possibly by induction of a conformational change in the region of the peptidyl transferase center caused by the binding of the aminoacyl-tRNA. However, this presumed conformational change is not possible during the stripping reaction exhibited by modified substrates (oligomers) employed here. Therefore, this structure-dependent discrimination

may well be one of the ways by which non-mRNA specified aminoacyl-tRNAs are excluded from acting as peptide chain acceptors during protein synthesis even though they possess the reactive aminoacyladenosine end.

In addition, these studies further emphasize the limitations of experiments in vitro, particularly with regard to extrapolation of results to the situations in vivo. Results from a number of laboratories (see Introduction, structural requirements for acceptor activity) have shown a wide variation in the acceptor substrate activity of many low-molecular weight analogues of aminoacyl-tRNA (both aminoacyl and nucleotidyl analogues of puromycin and aminoacyl and nucleotidyl derivatives of adenosine), depending on the assay system used and it is not known which assay system most closely resembles the situation in vivo. The present data on the reactivity of monomer-, 4mer- and 19mer-Tyr in washed polysome preparation and in "fragment reaction" on washed ribosomes reveal this point quite clearly. The results confirm and support the earlier suggestion by Pestka (1972) that the polyribosomes most closely mimic the situation in vivo and therefore should be exploited for a thorough understanding of the mechanism of action of peptidyl transferase. The suggestion was earlier supported by the observation that the structure-activity pattern of acceptor compound tested on polysomes more closely resembled the pattern of the complex cell-free systems (Symons et al., 1969; Harris et al., 1971).

Although the 'fragment reaction' is a simple and convenient way of assaying peptide bond synthesis, it cannot be used for a fair comparison of reactivity between bigger substrate molecules. This weakness of the 'fragment reaction' is most certainly related to the presence of 30%

methanol in this system. Although the specific effect of methanol (and other low molecular weight alcohols) in promoting the fragment reaction is most probably indicated by its enhancement of fragment binding (see Figure 26), its disordering effect on the structure of water may weaken the hydrophobic bidning of the acceptor substrates to the A site. Therefore, the conditions of the fragment reaction impose fairly severe limitations on the structural requirement for activity (Eckermann et al., 1974) relative to other assay systems where alcohol is not required.

CHAPTER V

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