

RIBOSOME-CATALYZED ESTER FORMATION

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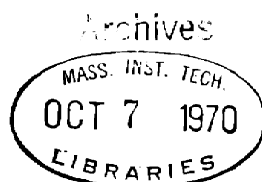
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ABSTRACT

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Submitted to the Department of Biology
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Ribosomal peptidyl transferase from *Escherichia coli* is able to catalyze transesterification reactions with the participation of α -hydroxyacyl acceptors. This is demonstrated with analogs of puromycin in which the α -amino group is replaced by a hydroxyl, and with α -hydroxyacyl-transfer RNAs, which are formed from aminoacyl-tRNAs by treatment with nitrous acid.

Peptidyl transferase transfers N-formylmethionine from fMet-tRNA or the terminal hexanucleotide fragment of fMet-tRNA to the α -hydroxyl groups of the puromycin analogs. Like the puromycin reaction, the reaction of the analogs is catalyzed by ribosomes, requires Mg^{2+} and K^+ ions, and is inhibited by chloramphenicol and gougerotin. In addition, the participation of peptidyl transferase is indicated by the similar pH dependence observed for the analog reaction and the puromycin reaction, and the fact that incubation of ribosomes at high temperatures destroys the ability to catalyze both reactions to the same degree.

The product of the puromycin analog reaction is an ester as shown by its lability under mildly alkaline conditions. It is otherwise indistinguishable from fMet-puromycin.

The ability of peptidyl transferase to use α -hydroxyacyl acceptor substrates is also demonstrated using α -hydroxyacyl-tRNAs. When phenyllactyl-tRNA, formed by treating phenylalanyl-tRNA with nitrous acid, is incubated in a ribosomal system containing polyuridylic acid, phenyllactic acid is incorporated into an alkali-labile, acid-precipitable product which appears to be

a polyester. The incorporation of phenyllactic acid, like the incorporation of phenylalanine in a similar *in vitro* system, requires ribosomes, poly U, GTP, and supernatant factors, and is inhibited by the protein synthesis inhibitors, chloramphenicol and gougerotin.

The incorporation of α -hydroxy acids into internal positions in a polypeptide is demonstrated in an *in vitro* system in which the RNA of an amber mutant of bacteriophage R17, a natural messenger RNA, directs the synthesis of a fragment of the phage coat protein. If an α -hydroxyacyl-tRNA is substituted for the corresponding aminoacyl-tRNA in the *in vitro* system the resulting peptide contains an alkali-labile ester linkage at a specific site which is determined by the position of the parent amino acid in the coat protein. These experiments indicate that peptidyl transferase of a single ribosome can make both ester and peptide linkages, and that it can do so while carrying out accurate translation of a natural mRNA.

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BIOGRAPHICAL NOTE

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TABLE OF CONTENTS

ABSTRACT	2
ACKNOWLEDGEMENT	4
BIOGRAPHICAL NOTE	5
TABLE OF CONTENTS	6
LIST OF FIGURES	9
LIST OF TABLES	12
INTRODUCTION	13
A. Chain Initiation	14
B. Chain Elongation	16
C. Chain Termination	20
D. Peptidyl Transferase	22
PUROMYCIN ANALOG REACTIONS	31
A. Introduction	32
B. Abbreviations	37
C. Materials	38
1. Miscellaneous	38
2. N-formylmethionine Methylester	38
3. Puromycin Analogs	39
D. Methods	41
1. Preparations	41
a. (^{35}S) Methionine	41
b. (^{35}S) fMet-tRNA	41
c. Hexanucleotide Fragment	42
d. Ribosomes	43
e. Initiation Factors	44

TABLE OF CONTENTS, Continued

2. Incubation Systems	45
a. fMet Fragment Reaction	45
b. fMet-tRNA Reaction	46
3. Electrophoretic Analysis	47
4. Liquid Scintillation Counting	47
E. Results	49
1. Reaction of ψ Hydroxypuromycin with the fMet Fragment	49
2. Characterization of the Product	64
3. The Nature of the Catalytic Unit	70
4. Reaction of fMet-tRNA	84
5. Position of the Reactive Hydroxyl	92
6. Nitrous Acid Deaminated Puromycin	111
F. Discussion	120
REACTIONS OF α -HYDROXYACYL-tRNAs	124
A. Introduction	125
B. Abbreviations	130
C. Materials	131
D. Methods	133
1. Preparations	133
a. Salt-washed Ribosomes	133
b. S-100	133
c. Native Ribosomal Subunits	133
d. RNA-free S-100	133

TABLE OF CONTENTS, Continued

e. Aminoacyl-tRNAs	134
f. Bacteriophage R17 RNA	144
2. Poly U-Directed System	147
3. Bacteriophage R17 RNA-Directed System	148
4. Interaction between T Factor and Phelac-tRNA	152
5. Double Label Counting	154
E. Results	156
1. The Poly U-directed System	156
a. The Incorporation of Phelac	157
b. Alkaline Lability	174
c. Number of Phelac Residues per Chain	181
2. The Phage R17 RNA-Directed System	191
3. T Factor Recognition of Phelac-tRNA	205
F. Discussion	212
DISCUSSION	217
A. Synthesis of Esters <i>in vivo</i>	218
B. The Catalytic Mechanism of Peptidyl Transferase	220
BIBLIOGRAPHY	225

LIST OF FIGURES

		<u>Page</u>
Figure 1	The reactions of formylmethionyl-tRNA with puromycin and demethoxy- α -hydroxypuromycin	33
Figure 2	Time course of the fMet fragment reaction with ψ hydroxypuromycin and puromycin	50
Figure 3	Dependence of the initial rate of the fMet fragment reaction upon concentration of ψ hydroxypuromycin and puromycin	56
Figure 4	Effect of fMet-fragment concentration on the kinetic parameters for puromycin	59
Figure 5	Dependence of the initial rate of the fMet fragment reaction upon concentration of DL- ψ hydroxypuromycin	62
Figure 6	Electrophoretic characterization of the products formed in the fragment reaction with ψ hydroxypuromycin	65
Figure 7	Alkaline hydrolysis of the fragment reaction products	68
Figure 8	Heat inactivation of ribosomes	71
Figure 9	Inhibition of the puromycin reaction by ψ hydroxypuromycin	75
Figure 10	Rate of the fragment reaction of puromycin and ψ hydroxypuromycin as a function of pH	79
Figure 11	Effect of pH on the kinetic parameters of puromycin	82

	<u>Page</u>
Figure 12 Electrophoretic characterization of the product of reaction between fMet-tRNA and ψ hydroxypuromycin	87
Figure 13 Alkaline hydrolysis of fMet- ψ hydroxypuromycin	90
Figure 14 Structures of the puromycin analogs . .	94
Figure 15 Time course of the fragment reaction in the presence of deaminopuromycin . .	97
Figure 16 Analysis of the ethyl acetate-extractable products formed in the presence of deaminopuromycin and N-acetylpuromycin	99
Figure 17 Inhibition of the puromycin reaction by deaminopuromycin	102
Figure 18 Inhibition of the puromycin reaction by N-acetylpuromycin	106
Figure 19 Inhibition of the puromycin reaction by α -chloropuromycin	109
Figure 20 Dependence of the fragment reaction rate on the concentration of α -hydroxypuromycin	114
Figure 21 Electrophoretic characterization of the product formed in the fragment reaction with α -hydroxypuromycin	117
Figure 22 Nitrous acid deamination of Phe-tRNA and Ala-tRNA	127
Figure 23 Chromatographic analysis of nitrous acid deaminated Phe-tRNA	137
Figure 24 Chromatographic analysis of nitrous acid deaminated Ala-tRNA	141
Figure 25 Ribonuclease digestion of Phelac-tRNA	149

	<u>Page</u>
Figure 26 Time course of the incorporation of Phelac	158
Figure 27 Effect of hot trichloroacetic acid treatment on the Phelac incorporation product	160
Figure 28 Analysis of the incorporated radio- activity after acid hydrolysis	163
Figure 29 Digestion of the Phelac incorporation product by α -chymotrypsin	164
Figure 30 Dependence of the incorporation of Phelac upon the concentration of ribosomes	166
Figure 31 Dependence of the incorporation of Phe and Phelac on the concentration of Magnesium ions	170
Figure 32 Alkaline hydrolysis of the Phelac incorporation product	175
Figure 33 Analysis of the incorporated radio- activity after alkaline hydrolysis	177
Figure 34 Outline of the R17 coat hexapeptide experiment	194
Figure 35 Purification of the hexapeptide products	196
Figure 36 Characterization of the hexapeptide products after alkaline treatment	199
Figure 37 Electrophoretic mobilities of peptides	202

LIST OF TABLES

		<u>Page</u>
Table 1	Requirements for reaction of the fMet-fragment with ψ hydroxypuromycin and puromycin	53
Table 2	Requirements for reaction of fMet-tRNA with ψ hydroxypuromycin	86
Table 3	Requirements for reaction of the fMet-fragment with α -hydroxypuromycin	113
Table 4	Kinetic parameters of the puromycin analogs	123
Table 5	Dependence of the incorporation of Phelac on GTP	168
Table 6	Inhibition of the incorporation of Phelac by chloramphenicol and gougerotin	172
Table 7	Alkaline stability of Phelac incorporated in the presence of Phe	182
Table 8	Number of Phelac residues per chain, according to Hervé and Chapeville	184
Table 9	Determination of the number of Phelac residues per chain	185
Table 10	Interaction of Phelac-tRNA with T_u	208
Table 11	Dependence of the binding of Phelac-tRNA to ribosomes on T_u and T_s	210

INTRODUCTION

INTRODUCTION

Protein biosynthesis in *Escherichia coli* can be arbitrarily divided into three more or less distinct processes:

(1) chain initiation, (2) chain elongation, and (3) chain termination. Chain initiation involves the assembly of an initiation complex from ribosomal subunits, messenger RNA (mRNA) and N-formylmethionyl-transfer RNA (fMet-tRNA). Chain elongation is the repeating process by which amino acids are added one at a time to the nascent polypeptide, proceeding from the amino terminus to the carboxyl terminus. Chain termination is the process by which the completed polypeptide chain is released from the ribosome. (For general references, see the recent review of protein biosynthesis by Lengyel and Söll (1969). Ribosome structure and metabolism have recently been reviewed by Schlessinger and Apirion (1969). In addition there is a number of useful review articles in the Cold Spring Harbor Symposia on Quantitative Biology, Volume 34 [1969].)

Chain Initiation

The initiation of polypeptide chains has been shown in cell free protein synthesizing systems using natural mRNA to proceed with the incorporation of N-formylmethionine at the N-terminus (Adams and Capecchi, 1966; Webster, et al, 1966; Clark and Marcker, 1966). The process requires the

assembly of an "initiation complex" in which fMet-tRNA and mRNA are bound to the ribosome. The nature of the chain initiation signal on mRNA is not entirely understood, in spite of the fact that the mRNA sequences involved in the formation of the initiation complex for several RNA phage proteins are known (Steitz, 1969; Steitz, 1969b; Hindley and Staples, 1969; Gupta, et al, 1970). All have the triplet AUG, which specifies fMet. However, this triplet cannot by itself constitute the initiation signal, since the same triplet specifies methionine in internal positions (Adams, et al, 1969; Tsugita, et al, 1969). Since a physical end of the message is not required (Bretscher, 1969) the initiation signal must involve more than a single triplet. It is likely that tertiary structure of the mRNA is important (Lodish, 1969).

The initiation complex is assembled from mRNA, fMet-tRNA, and the 30S and 50S ribosomal subunits (Guthrie and Nomura, 1968) with the participation of at least three protein factors (Brawerman, 1969; Dubnoff and Maitra, 1969; Revel, et al, 1969; Thach, et al, 1969; Wahba, et al, 1969). Though there is some disagreement among these groups as to the roles of the individual factors, the following is a reasonable consensus. (Using the nomenclature of Wahba, et al, [1969].) F_3 is involved in the binding of

the initiator region of mRNA to the 30S ribosomal subunit. F_2 is also involved in the binding of mRNA to the 30S subunit, and is required for the binding of fMet-tRNA. F_1 is required for addition of the 50S subunit to the 30S complex to form the 70S complex.

In addition to these factors, formation of the initiation complex requires GTP, which is hydrolyzed during the process (Hershey and Thach, 1967; Thach, et al, 1967). fMet-tRNA bound to ribosomes in the presence of GTP is able to participate in peptide bond formation by transferring fMet to puromycin (see below) or to aminoacyl-tRNA. This functional criterion defines the ribosomal site at which it is bound as the donor site (D site).

Chain Elongation

Once the 70S initiation complex is formed with fMet-tRNA in the donor site, a site is made available for aminoacyl-tRNA binding, specified by the next triplet (in the 3'direction) on mRNA (Erbe, et al, 1969). The binding of aminoacyl-tRNA to the ribosome requires the participation of two protein factors, T_U and T_S , plus GTP (Ravel, 1967; Erbe, et al, 1969; Ertel, et al, 1968; Lucas-Lenard, 1968). The actual species which binds to the ribosome is a ternary complex of aminoacyl-tRNA, T_U , and GTP. The formation of the complex requires T_S , which is released from the complex before it binds to the ribosome (Shorey, et al, 1969). A

ternary complex can also be formed with the β - γ methylene analog of GTP and such a complex can also bind to ribosomes, but the amino acid cannot be incorporated into a polypeptide (Haenni and Lucas-Lenard, 1968). Thus the active ribosome binding of aminoacyl-tRNA requires the hydrolysis of GTP, at some stage subsequent to the physical binding. A T_U -GDP complex is released (Shorey, et al, 1969). This GTP hydrolysis may be involved in shifting aminoacyl-tRNA from one binding site to another, (This would define an "entry site.") or activation by a conformational change of tRNA or of the ribosome. Whether or not a distinct entry site exists on the ribosome, only one molecule of aminoacyl-tRNA can be bound, in addition to fMet-tRNA, to the initiation complex (Roufa, et al, 1970).

The ternary T-factor complex cannot be formed with deacylated tRNA (Gordon, 1967; Shorey, et al, 1969) or with fMet-tRNA (Ono, et al, 1968). T-factor therefore serves the important function of excluding unwanted species of tRNA from the A site during chain elongation.

A peptide bond is then formed by the transfer of fMet to the α -amino group of aminoacyl-tRNA. This reaction defines the "acceptor site" (A site) on the ribosome. The product of the reaction is a dipeptidyl-tRNA which is presumably still bound in the A site. (It is not reactive with puromycin.) The shift of peptidyl-tRNA into the D site requires the participation of a non-ribosomal protein factor,

G, and the hydrolysis of GTP (Erbe, et al, 1969; Brot, et al, 1968). The nature of this process, called translocation, is not understood. Translocation also brings about the release of deacylated tRNA from the D site (Kuriki and Kaji, 1968; Lucas-Lenard and Haenni, 1969).

Thus, after translocation, peptidyl-tRNA is located at the D site and the A site is vacant. The situation is analogous to the initiation complex, with dipeptidyl-tRNA substituted for fMet-tRNA and the mRNA shifted one codon. With each repeat of the sequence just outlined of aminoacyl-tRNA binding, peptide bond formation, and translocation, the polypeptide chain is elongated by one amino acid and the ribosome moves one codon along the mRNA.

During the chain elongation process, as elucidated in dissected form in *in vitro* systems, two molecules of GTP are hydrolyzed to GDP and inorganic phosphate per peptide bond. The stoichiometry of the entire process has been reported to be one GTP split per peptide bond (Nishizuka and Lipmann, 1966). Because of a large "background" hydrolysis which was subtracted by Nishizuka and Lipmann, but which may have been relevant, it is possible that this value is too low by a factor of two. Nevertheless, this disagreement emphasizes the fact that *in vitro* results on fractionated systems must be interpreted with caution. It is particularly interesting in this regard that when the transfer factors

are purified from *E. coli* in the presence of a proteolytic inhibitor, both T and G activities are found in a single component which is in itself sufficient for *in vitro* protein synthesis (Hollis and Furano, 1968). It is possible that the nature of the process which is studied using separated and purified factors differs in some important ways from the *in vivo* process.

The terms "A site" and "D site" imply distinct geometrical loci to which tRNA binds. They are, however, defined by functional criteria, and in fact represent functional states of tRNA. The question whether each functional state is synonymous with a physical location on the ribosome is unresolved. This is another way of saying that the nature of the process of translocation is not understood. According to the most popular model, on which the term site is based, and which has been implicit in the preceding discussion, the A and D sites are two separate locations on a 70S ribosome (Gilbert, 1963; Watson, 1964). A model which is conceptually related, but in which the two ribosomal subunits move relative to one another with resulting changes in the nature of the sites, has been proposed by Bretscher (1968b). A very different model, which is mostly of interest because it points out that the above kinds of models do not uniquely account for what is known about protein synthesis, has been proposed by Woese (1970). According to this model there

are two functionally identical ribosomal sites for binding tRNA. The A site-D site functional distinction is based on the conformation of the anticodon loop of tRNA. Once bound to the ribosome a molecule remains in a single location until it is released. The process of translocation involves a change in the conformation of the anticodon loop which moves the mRNA to which it is hydrogen bonded through a distance of exactly three nucleotides.

Chain Termination

During protein synthesis the nascent polypeptide chain remains esterified through its carboxyl group to the terminal adenosine residue of a molecule of tRNA (Gilbert, 1963; Bretscher, 1965). Once completed, the protein is released from the ribosome by hydrolysis of the peptidyl-tRNA ester bond.

If one of three "nonsense" triplets (UAA, UAG, or UGA) is inserted by mutation into a cistron in proper phase, it brings about premature termination of the polypeptide chain (Stretton and Brenner, 1965; Zinder, et al, 1966, Suzuki and Garen, 1969; Sambrook, et al, 1967). Evidence has recently been accumulating which indicates that these triplets are involved in the normal chain termination process as well. The mRNA sequence at the end of the phage R17 coat protein cistron contains, following the codon which specifies the carboxyl terminal amino acid, the

sequence UAAUAG (Nichols, 1970). A frameshift mutation in the *hisD* gene of *Salmonella typhimurium* has allowed the determination that the normal chain termination codon is either UAA or UGA (Rechler and Martin, 1970). In suppressor strains of *E. coli* which insert tyrosine in response to the codons UAA and UAG, extra tyrosine residues are found at the carboxyl terminus of a mixture of total proteins (Lu, 1970).

The process of chain termination directed by the three nonsense triplets has been studied *in vitro* using the RNA of an amber (UAG) mutant of bacteriophage R17 (Capecchi, 1967b). Chain termination in that system requires the presence of a protein factor, R. Using the trinucleotide codons, Scolnick, et al, (1968) resolved R into two separate components, one of which (R_1) mediates termination directed by the triplets UAA and UAG, and the other (R_2) responds to UAA and UGA. In addition, a third factor (S) stimulates the rate of termination in the presence of R_1 or R_2 in that system (Milman, et al, 1969). The presence of these factors which function specifically in response to the nonsense triplets further suggests that these triplets are functional *in vivo*. In addition, Capecchi and Klein (1970) have shown that either R_1 or R_2 can mediate the termination of the complete coat protein or RNA synthetase of phage R17 *in vitro*. This implies that the terminator codon for both cistrons is UAA, in agreement with the known sequence

for the coat protein (Nichols, 1970).

The protein factors are involved in the recognition of the chain termination signal. The actual hydrolysis of the peptidyl-tRNA ester bond in response to this signal appears to be catalyzed by the same ribosomal unit which catalyzes the formation of peptide bonds (Vogel, et al, 1969; Capecchi and Klein, 1969; Tompkins, et al, 1970). This conclusion is based on the fact that the termination reaction is inhibited by a number of compounds which also inhibit peptide bond formation but do not inhibit tRNA binding to ribosomes.

Upon release of the completed protein from tRNA, the ribosome-mRNA complex is rapidly dissociated (Webster and Zinder, 1969) presumably releasing 30S and 50S subunits (Kaempfer, et al, 1968). Additional protein factors may be required for the release of the deacylated tRNA which remains on the ribosome (Kaji, et al, 1969) and/or dissociation of the 70S particle into subunits (Subramanian, et al, 1968). The latter factor may be identical to one of the "initiation factors" discussed above.

Peptidyl Transferase

The formation of peptide bonds during protein synthesis is catalyzed by an enzyme which is an integral part of the 50S ribosomal subunit (Monro, 1967; Maden, et al, 1968; Gottesman, 1967). Thus, addition of a single amino

acid to a polypeptide can be observed on ribosomes which have been extensively washed with high concentrations of salt, in the absence of all of the extraribosomal factors described above and without any nucleoside triphosphate (Gottesman, 1967).

The normal reaction catalyzed by peptidyl transferase during protein synthesis consists of transfer of the carboxyl group of the nascent polypeptide chain (or fMet) from peptidyl-tRNA to the α -amino group of aminoacyl-tRNA. In addition, peptidyl transferase can catalyze analogous transfer reactions in which the acceptor substrate is the antibiotic puromycin. The structure of puromycin is shown in Figure 1. It is an analog of the aminoacyl end of aminoacyl-tRNA (Yarmolinski and de la Haba, 1959).

Puromycin is able to bring about the release of incomplete polypeptide chains from the ribosome by being incorporated into the polypeptide at its carboxyl end, both *in vivo* (Nathans, 1964) and *in vitro* (Smith, et al, 1965). In addition it can react with ribosome-bound fMet-tRNA to form formylmethionyl-puromycin (see Figure 1) (Bretscher and Marcker, 1966; Zamir, et al, 1966). The evidence that these reactions of puromycin occur by the same mechanism as peptide bond formation during protein synthesis, and are catalyzed by the same enzyme, peptidyl transferase, has been summarized by Maden, et al, (1968)

as follows. (1) Puromycin is analogous to aminoacyl-tRNA and the product of the reaction is analogous to the normal product. Furthermore, only the L-phenylalanyl analog of puromycin is active, whereas the D-phenylalanyl analog is inactive. (2) The reaction is catalyzed by ribosomes. (3) The reaction is dependent upon monovalent and divalent cations (Maden and Monro, 1968), as is protein synthesis. (4) The puromycin reaction is inhibited by a number of specific inhibitors of protein synthesis, including chloramphenicol, gougerotin, and sparsomycin. All of these lines of evidence serve to establish a connection between the puromycin reaction and normal peptide bond formation, but the argument that peptidyl transferase is involved is based almost entirely on the structural similarity of the reactants and products to the normal ones.

The puromycin reaction has proved useful for examining the properties of peptidyl transferase as distinguished from the specific substrate binding functions of the ribosome. That is, it allows the examination of peptidyl transferase independently of the requirements for the binding of aminoacyl-tRNA to the ribosome, which is influenced by interactions with messenger RNA and T factor.

A relatively simple donor substrate for peptidyl transferase, with the same advantages, has been obtained

by Monro and Marcker (1967). They have demonstrated a reaction of puromycin with a formylmethionine-containing hexanucleotide (CAACCA-fMet) derived from fMet-tRNA by digestion with ribonuclease-T₁. The product of the reaction is formylmethionyl-puromycin. This reaction, which is referred to as the fMet fragment reaction, requires only the 50S ribosomal subunit, monovalent and divalent cations, and methanol or ethanol (Monro, 1967; Maden and Monro, 1968; Monro, et al, 1968). The requirement for alcohol has not been explained. It may be required for the binding of the hexanucleotide fragment to the ribosome or for some ribosomal conformation change which may be required to activate peptidyl transferase in the absence of most of the ribosome-tRNA interactions. It is of interest in this context that the R factor-mediated polypeptide chain termination reaction can take place in the absence of the appropriate codon, but under these circumstances ethanol is required (Milman, et al, 1969). As discussed above, it is likely that the termination reaction is also catalyzed by peptidyl transferase.

Because of the relatively simple structures of the substrates involved and the lack of mRNA, factor, and energy requirements, it is reasonable to assume that the fragment reaction involves only the minimal peptide bond forming unit. In other words, it is useful to define peptidyl

transferase as that unit which is involved in the catalysis of the fragment reaction. The usefulness of this definition depends on the assumption that peptide bond formation in the fragment reaction occurs by the same mechanism as peptide bond formation during protein synthesis. The lines of evidence in support of this assumption are the same as those concerned with the relevance of the puromycin reactions described above.

Peptidyl transferase was localized to the 50S subunit by the demonstration of a reaction between polyphenylalanyl-tRNA bound to the 50S subunit and puromycin (Maden, et al, 1968) and by the fragment reaction, which takes place in the presence of purified 50S subunits (Monro, 1967). In addition, the fragment reaction has been used to demonstrate inhibition of peptidyl transferase by a number of inhibitors of protein synthesis, including chloramphenicol, gougerotin, and sparsomycin (Monro and Vazquez, 1967).

Studies of the donor substrate specificity in the fragment reaction (Monro, et al, 1968) have indicated that the minimal fragment which is active is the aminoacyl terminal trinucleotide, CpCpA, of tRNA. The nature of the amino acid side chain is relatively unimportant, but the α -amino group must be acylated either with a formyl group or an acetyl group. This requirement of peptidyl transferase for a blocked amino group on the donor substrate suggests an explanation

for the use of N-formylmethionine as chain initiator in *E. coli*, even though the formyl group is not found in the finished protein (Walker, 1963; Konigsberg, et al, 1966). By analogy, the observation of a similar requirement for a blocked amino group in the fragment reaction catalyzed by eukaryotic (human tonsil) ribosomes (Neth, et al, 1970) implies that a N-blocked initiator of some sort is required in that system as well.

The structural requirements of the acceptor substrate have been studied using analogs of puromycin and various aminoacyl nucleosides which act analogously to puromycin (Waller, et al, 1966). The minimal substrate is an aminoacyl-nucleoside (Nathans and Neidle, 1963). The nucleoside portion of the acceptor substrate can be either adenosine, inosine, or cytosine, though adenosine is most effective. Guanosine and uridine are inactive. The 2'-hydroxyl group is important, since 2'-deoxyadenosine is less active than adenosine. The requirement for the 2'-hydroxyl is not absolute, however, since phenylalanyl-2'-deoxyadenosine is reactive to some extent (Rychlik, et al, 1969; Cerna, et al, 1970). I have verified the activity of phenylalanyl-2'-deoxyadenosine using a highly purified preparation containing very little phenylalanyl-adenosine (far too little to account for the activity). Furthermore, I have found that an analog of puromycin in which the

2'-hydroxyl group is in the opposite configuration (an arabinosyl analog) is also active, but only weakly. (These experiments are not included in this dissertation.)

Among aminoacyl nucleoside acceptor substrates only compounds with aromatic aminoacyl side chains are active (Nathans and Neidle, 1963; Symons, et al, 1969). However, the inactive L-glycyl analog of puromycin or L-glycyl-adenosine becomes nearly as active as puromycin when a cytidine-3'-phosphoryl residue is attached to its 5'-hydroxyl group (Rychlik, et al, 1967; Symons, et al, 1969). Thus it appears that the peptidyl transferase acceptor site recognizes the terminal dinucleoside of tRNA, but that in the presence of an aromatic aminoacyl side chain the interactions with the cytidyl residue are not essential.

The configuration at the α -carbon of the amino acid is also recognized by peptidyl transferase. The amino acid must be of the L-configuration. In addition it must be attached at the 3'-position of the nucleoside (Nathans and Neidle, 1963). Though the amino acid in aminoacyl adenosine (ester) equilibrates between the 2' and 3' positions (Rammler and Khorana, 1963; Wolfenden, et al, 1964) the fact that the 2' analog of puromycin is inactive implies that only 3'-aminoacyl-tRNA is active. Experiments by Cathey and Klebanoff (1967), using an *in vitro* system derived from *Lactobacillus acidophilus* which is active in

protein synthesis below pH 6, indicated that puromycin is only active if its α -amino group ($pK_a = 7.3$ [Nathans, 1967]) is not ionized. This fact is of importance in relation to the activity of α -hydroxyacyl acceptor substrates which is the subject of this dissertation.

While some of the structural requirements of the substrates reflect the interactions involved in binding the substrates to the peptidyl transferase active site, they may in addition have more elaborate functions. For example, phenylalanyl-2'-deoxyadenosine is less active in the fragment reaction than is phenylalanyl-adenosine (Cerna, et al, 1970). While this decreased activity may in part reflect a lower affinity of the enzyme for the 2'-deoxy compound, this rate is much lower than the reaction rate with phenylalanyl-adenosine, even at high concentrations of the substrate, where the rate of reaction is independent of the substrate concentration. The same behavior has been observed with the arabinosyl analog of puromycin (Unpublished observation). This suggests that the 2'-hydroxyl group performs some important function in the catalysis of the reaction. Since some activity is observed with the deoxy compound, the 2'-hydroxyl does not obligatorily participate in the formation of an intermediate bis(2'-peptidyl-3'-aminoacyl)ester, as suggested by Neumann, et al, (1968). Instead, it may function

by inducing some conformational change in the enzyme or by participating directly (for example, as a hydrogen bond donor) in the formation of the transition state.

Another example, which may be related, has been reported by Scolnick, et al, (1970). They have shown that in the presence of ethanol, the ethyl ester of fMet can be formed from fMet-tRNA, presumably in a reaction catalyzed by peptidyl transferase. This reaction only takes place in the presence of the trinucleotide CpCpA. Furthermore, if the 3'-terminal ribose of the trinucleotide has been oxidized with periodate it is no longer active. Thus, the 2'- and/or 3'-hydroxyls appear to be involved in the stimulation of the reaction. This phenomenon may be related to the decreased activity of 2'-deoxy acceptor substrates. Both of these observations can be interpreted in terms of a model wherein peptidyl transferase can exist in an inactive state. Its activation might require recognition of some ribosome-substrate interactions, perhaps involving the adenosine moiety of the acceptor substrate and some other tRNA-ribosome-mRNA interactions. The requirements for CpCpA in the ethanol reaction, for release factors in the process of chain termination, and for alcohol in the fragment reaction can be interpreted in terms of such a model.

PUROMYCIN ANALOG REACTIONS

In the experiments reported in this section the puromycin reaction is used as a model system to examine the substrate structural requirements of *E. coli* ribosomal peptidyl transferase. The observation is made that an analog of puromycin in which the α -amino group is replaced by a hydroxyl group can participate in a ribosome catalyzed reaction with either N-formylmethionyl-tRNA or the terminal fMet containing hexanucleotide fragment of fMet-tRNA.

The α -hydroxyl analog of puromycin was synthesized from puromycin aminonucleoside and L- β -phenyllactic acid. Its structure is shown in Figure 1. It differs from puromycin in two ways: The α -amino group of puromycin is replaced by a hydroxyl and the p-methoxy group on the aminoacyl portion of puromycin is missing. The compound will be referred to as demethoxy- α -hydroxypuromycin, abbreviated ψ hydroxypuromycin. A compound which behaves similarly to ψ hydroxypuromycin in the *in vitro* systems described here is formed from puromycin by nitrous acid treatment. This compound will be referred to as α -hydroxypuromycin. Its structure is presumably the same as that of ψ hydroxypuromycin except for the presence of the p-methoxy group. The p-methoxy group does not appreciably affect the activity of puromycin (Nathans and Niedle, 1963).

FIGURE 1: THE REACTIONS OF N-FORMYLMETHIONYL-
tRNA WITH PUROMYCIN AND DEMETHOXY-
 α -HYDROXYPUROMYCIN.

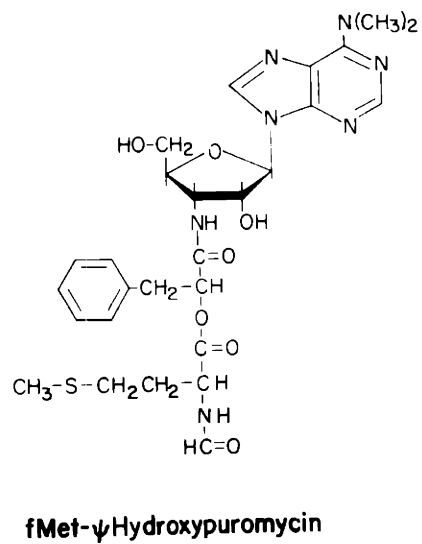
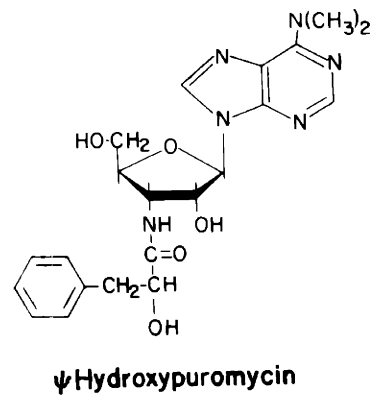
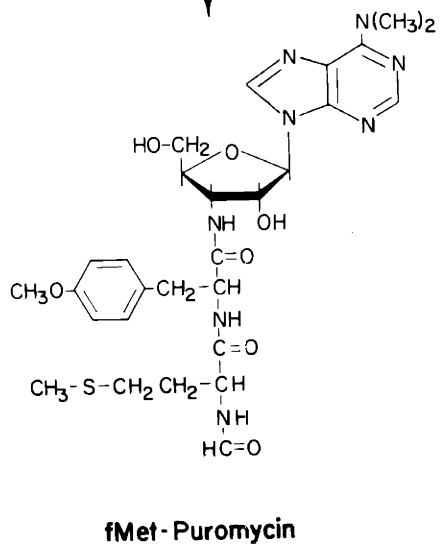
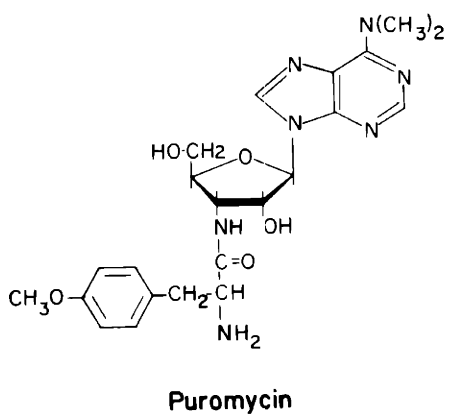


Figure 1

The product of reaction between ψ hydroxypuromycin and fMet-tRNA or the fMet fragment resembles fMet-puromycin, which is formed under the same conditions in the presence of puromycin (Figure 1). It differs from fMet-puromycin by its lability under mildly alkaline conditions since fMet is joined to ψ hydroxypuromycin through a labile ester linkage instead of the stable amide linkage present in fMet-puromycin.

A variety of similarities between the reactions of puromycin and ψ hydroxypuromycin indicate that both reactions are catalyzed by the same enzyme, namely ribosomal peptidyl transferase.

The reaction with the fMet-fragment was studied most extensively because of its simplicity. The disadvantage of this system in terms of interpreting the physiological relevance of the results is that the reaction requires the presence of methanol or ethanol. However, this requirement is also an advantage in these experiments since methanol enhances the solubility of the puromycin analogs, allowing the use of concentrations which would be beyond saturation in aqueous solutions.

The activity of ψ hydroxypuromycin is not limited to the fragment system and high concentrations of methanol. The analog also reacts with fMet-tRNA bound to ribosomes in the presence of messenger RNA (bacteriophage R17 RNA),

initiation factors, and GTP. This reaction requires no methanol.

It is therefore concluded that peptidyl transferase has the capacity to make use of an α -hydroxyacyl acceptor substrate to form an ester linkage in addition the peptide bond formation which is observed with α -aminoacyl acceptors.

ABBREVIATIONS

ATP -- adenosine-5'-triphosphate, sodium salt

CTP -- cytidine-5'-triphosphate, sodium salt

DNase -- bovine pancreatic deoxyribonuclease I

EDTA -- ethylene diamine tetraacetic acid

GTP -- guanosine-5'-triphosphate, sodium salt

HEPES -- N-2-hydroxyethylpiperazine-N'-2-ethane
sulfonic acid

Met -- L-methionine

fMet -- N-formyl-L-methionine

RNA -- ribonucleic acid

tRNA -- transfer ribonucleic acid

RNase -- bovine pancreatic ribonuclease

Tris -- tris-(hydroxymethyl)-aminomethane

MATERIALS

Puromycin was obtained from Cyclo Chemical Company and Nutritional Biochemicals. Chloramphenicol was a gift from Parke, Davis and Company. Gougerotin was a gift from Takeda Chemical Industries, Limited, Japan. Tris and ATP were purchased from Sigma Chemical Company. Glutathione (reduced), GTP, CTP, creatine phosphate (sodium salt), creatine phosphokinase, and HEPES were purchased from Calbiochem. N-formyl-DL-methionine and methionine methylester were obtained from Mann Research Laboratories, Incorporated. Folinic acid (calcium salt) and *E. coli* K12 tRNA were obtained from General Biochemicals. R17 RNA was a gift from P. Lu. Ribonuclease T₁, pancreatic RNase, and electrophoretically purified DNase were obtained from Worthington Biochemical Corporation. Levigated alumina, abrasive grain, was obtained from Norton Company, Worcester, Massachusetts. Sephadex G25 (50 to 150 μ particles) was purchased from Pharmacia Fine Chemical AB, Uppsala.

N-formylmethionine methylester was obtained by chemical formylation of methionine methylester (Sheehan and Yang, 1958). Twenty-six mg DL-methionine methylester was dissolved in 2.5 ml 90% formic acid. Acetic anhydride (0.83 ml) was added dropwise with stirring, and the solution

was stirred at room temperature for 1 hour. It was then dried on a Büchi flash evaporator and the residue shaken with 1 ml aqueous 0.3 M sodium acetate, pH 5.5, and 1 ml ethyl acetate. The ethyl acetate layer was removed and the aqueous phase reextracted with 1 ml ethyl acetate. The combined ethyl acetate layers were dried on a flash evaporator.

Puromycin Analogs

Demethoxy- α -hydroxypuromycin (abbreviated ψ hydroxypuromycin), deaminopuromycin, N-acetylpuromycin, α -chloropuromycin, and α -methoxypuromycin were prepared and chemically characterized by Dr. H. Neumann. All were synthesized from puromycin aminonucleoside and the appropriate carboxylic acid, except N-acetylpuromycin which was obtained by acetylation of puromycin. The α -substituted acids used in the synthesis of α -chloropuromycin and α -methoxypuromycin were racemic D, L mixtures.

ψ hydroxypuromycin, the most important analog in these experiments was synthesized by condensation of L-(-)-3-phenyllactic acid (Aldrich Chemical Company) and puromycin aminonucleoside (Sigma Chemical Company) in the presence of dicyclohexyl carbodiimide. The crystalline synthetic product was characterized and its structure proved by nuclear magnetic resonance, ultraviolet, and

infrared spectroscopy.

α -hydroxypuromycin was obtained by nitrous acid deamination of puromycin. One hundred mg puromycin dihydrochloride^d_A was dissolved in 20 ml cold (0° C) 1 N HCl. To this was added, slowly with stirring, 20 ml cold 1 N NaNO₂. The mixture was incubated overnight at 0° C. Four ml 5 N NaOH was then added, and the mixture extracted with three 50 ml aliquots of ethyl acetate. Combined ethyl acetate layers were washed with 25 ml H₂O and dried at room temperature on a Büchi flash evaporator. The material was dissolved in methanol and subjected to chromatography on a 2 mm thick layer of Silica Gel G with fluorescent indicator (Analtech) in carbon tetrachloride/methanol (10:1). Ultraviolet absorbing bands were located and eluted with acetone. Three bands were obtained, none of which corresponded to puromycin. The band pattern was not appreciably different if the deamination was carried out under conditions similar to those of Hervé and Chapeville (1965). Only one of these bands proved to be active in the fragment system as described below. It was a minor component comprising about 10% of the total.

METHODS

(³⁵S) methionine of specific activity 1,000 to 5,000 mC/mmole was prepared by the method of Sanger, et al, (1964). Baker's yeast was grown in a medium containing carrier free ³⁵SO₄²⁻, the proteins digested with a mixture of pronase (Calbiochem) and Worthington protease, and methionine purified by paper chromatography.

The product was used to prepare (³⁵S) fMet-tRNA by the procedure of Hershey and Thach (1967). The charging mixture contained, per ml: 2 mg *E. coli* K12 stripped tRNA; 0.1 M Tris-HCl, pH 7.1; 0.02 M magnesium acetate; 7.5 mM ATP, 0.2 mM CTP, 20 mM creatine phosphate; 70 µg creatine phosphokinase; 0.6 mM folinic acid; 10 mM glutathione; 0.03 mM each, 19 unlabelled amino acids (minus Met); 30 µC (³⁵S) Met; and 3 mg S-100 protein. After 20 min incubation at 37° C the pH was lowered to about 5 with acetic acid, 22 µmoles EDTA was added, and the protein was extracted by vigorous mixing for 10 min. at 2° C with 1 ml vacuum-distilled, water-saturated phenol. The phases were separated by centrifugation at 18,000 Xg for 15 min. in a Servall SS-1 rotor at 2° C. To the aqueous phase was added 0.1 volume 20% w/v potassium acetate, pH 5, and 2 volumes cold ethanol. After 1 hour at -20° C, the precipitate was

collected by centrifugation and redissolved in 0.5 ml cold water. This solution was then passed through a 0.7 cm × 20 cm column of Sephadex G25 (Pharmacia), equilibrated with water, at 2° C. The first peak of UV absorbing (260 nm) material was collected and precipitated with 0.1 volume 20% potassium acetate plus 2 volumes cold ethanol. The precipitate was collected and washed with cold (-20° C) ethanol. Ethanol was then removed under vacuum, and the residue was dissolved in water and stored frozen at -20° C.

The (^{35}S) fMet containing hexanucleotide fragment of fMet-tRNA was prepared as described by Marcker (1965), by digestion with RNase T₁. Six mg (^{35}S) fMet-tRNA was incubated with 100,000 units RNase T₁ (Worthington) in 1 ml 0.01 M sodium acetate, pH 5.4, 0.001 M EDTA, for 30 min. at 37° C. The solution was lyophilized and the residue taken up in a small volume of 0.08 M ammonium formate buffer, pH 3.5. The digest was subjected to electrophoresis on Whatmann 3 MM paper for 4 hours at 37 V/cm. Radioactivity was located by means of a Vanguard gas flow strip counter. The resulting radioactivity distribution was similar to that of Marcker (1965). The major peak in the direction of the anode was eluted with 0.001 M ammonium formate, pH 3.5, 0.02 M β mercaptoethanol, and stored at -20° C. Digestion of the final product with pancreatic

RNase produced only fMet-adenosine.

E. coli Q13, which is deficient in RNase I, was grown from a stock provided by W. Gilbert. Cultures (15 l) were grown to mid-log phase at 37° C with aeration in a medium composed of 10 g Bacto Tryptone (Difco), 5 g yeast extract (Difco), 5 g NaCl, and 1 ml 1 N NaOH per liter, then quickly chilled by adding an excess of ice. Cells were harvested over about 1 hour by centrifugation through a Servall continuous flow apparatus, and washed with 0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl, 0.05 M KCl, 0.02 M NH₄Cl, 0.01 M MgCl₂. Packed cells were frozen in a dry ice-acetone bath and stored at -20° C. The usual yield was about 2 gm packed cells per liter medium.

Salt washed ribosomes and crude (unfractionated) initiation factors were prepared from *E. coli* Q13 by the procedure of Anderson, et al, (1967). All steps were carried out at 2° to 4° C. Thirty g freshly thawed cells were ground in an unglazed mortar with 75 g alumina, added gradually. The mixture was then suspended in 60 ml standard buffer (0.01 M Tris-HCl, pH 7.4, 0.01 M magnesium acetate, 0.06 M NH₄Cl, 0.006 M β -mercaptoethanol). Alumina and cell debris were removed by centrifugation for 20 min at 12,000 rpm in a Servall SS-1 rotor. DNase (0.1 ml, 0.1 mg/ml) was added and the extract incubated 30 min. at 0° C. The extract was then centrifuged for 10 min. at 33,000 rpm in

a Spinco 65 rotor. The upper 4/5 of the supernatant was removed and recentrifuged for 10 min. at 33,000 rpm. The upper 2/3 of the supernatant of this second spin was removed.

Ribosomes were pelleted by centrifugation for 3 hours at 60,000 rpm in a Spinco 65 rotor. The supernatant was removed within 1/2 cm of the pellet and used for preparation of S-100 as described below. Ribosome pellets (3) were resuspended in 5 ml each of standard buffer by overnight shaking on a wrist action shaker, and again pelleted by centrifugation for 3 hours at 60,000 rpm. Pellets were resuspended in 5 ml of buffer composed of 2 M NH_4Cl , 0.01 M Tris-HCl, pH 7.4, 0.01 M magnesium acetate. White precipitate was removed by low speed centrifugation, then the ribosomes were pelleted by centrifugation for 3 hours at 60,000 rpm. The supernatant from this centrifugation was used for preparation of initiation factors, as described below. The ribosomal pellets were washed twice more with a buffer composed of 0.5 M NH_4Cl , 0.01 M Tris-HCl, pH 7.4, 0.01 M magnesium acetate, by the above procedure. The washed ribosomes were finally resuspended in 4 ml standard buffer, frozen in dry ice, and stored in aliquots at -20°C . The final solution A_{260} was 1300. Analysis of the final product by zone sedimentation in a sucrose density gradient in 0.01 M magnesium revealed 70% 50S sub-

units, 20% 30S subunits, and 10% 70S ribosomes.

Preparation of initiation factors: To 32 ml supernatant from the 2 M ammonium chloride wash of ribosomes was added, slowly with stirring at 0° C, 16.5 g ammonium sulfate. The pH was maintained at 7.8 with KOH during addition of ammonium sulfate. After 30 min. at 0° C the precipitate was removed by centrifugation and redissolved in 3 ml buffer composed of 0.02 M Tris-HCl, pH 7.4, 0.002 M magnesium acetate, 0.18 M NH₄Cl, 0.006 M β -mercaptoethanol. The solution was dialyzed overnight against 2 \times 1 liter of the same buffer. Insoluble material was removed by centrifugation and the solution frozen in dry ice and stored in aliquots at -20° C.

Postribosomal supernatant (S-100) from the initial centrifugation was dialyzed for 18 hours against 3 \times 1 liter standard buffer frozen in dry ice and stored in aliquots at -20° C.

Protein concentrations were determined by the method of Lowry, et al, (1951) using as a standard bovine serum albumin (Armour Pharmaceutical Company).

Incubation Systems

fMet fragment Reaction (Monro and Marcker, 1967; Maden and Monro, 1968) -- Prior to methanol addition, the reaction mixture (0.1 ml) contained 0.06 M Tris-HCl buffer

(pH 8.1, measured at 0° C), 0.4 M KCl, 0.02 M magnesium acetate, 0.006 M β -mercaptoethanol, 13.8 A₂₆₀ units per ml salt washed ribosomes, and the formylmethionyl T₁ fragment, (50,000 dpm/ml, or as noted in figure legends).

The reaction was initiated with an equal volume of methanol (blank) or a methanolic solution of puromycin or puromycin analogs. Concentrations of puromycin and analogs were determined spectrophotometrically in 0.1 N HCl solution ($\lambda_{\text{max}} = 267.5 \text{ nm}$; $\epsilon = 2.0 \times 10^4$ for all compounds). Incubation was at 0° C for time periods noted in figure legends. The reaction was terminated with 25 μ l of 0.1 M BeCl₂, and 0.1 ml of 0.3 M sodium acetate (pH 5.5) saturated with MgSO₄, and then 1.5 ml of ethyl acetate were added. The mixture was shaken at room temperature for 15 sec. and centrifuged briefly. One ml of the ethyl acetate layer was counted in a liquid scintillation spectrometer.

fMet-tRNA reaction -- The complete reaction mixture contained 0.1 M Tris-HCl (pH 7.4); 0.005 M magnesium acetate; 0.05 M KCl; 0.006 M β -mercaptoethanol; 0.6 mM GTP; 0.93 A₂₆₀ units/ml (³⁵S) fMet-tRNA, containing 2.3×10^5 dpm/A₂₆₀ unit and about 60% formylated; 41 A₂₆₀ units per ml of ribosomes and 0.8 mg/ml of crude initiation factors; 7.2 A₂₆₀ units /ml of bacteriophage RL7 RNA; and 1.0×10^{-3} M ψ hydroxypuromycin. 0.1 ml aliquots were

incubated for 15 min. at 30° C. The reaction was terminated with 25 μ l of 0.1 M BeCl_2 . 0.1 ml of 0.3 M sodium acetate (pH 5.5) saturated with MgSO_4 and 1.5 ml ethyl acetate were added and the mixture was shaken at room temperature for 15 sec. Following brief centrifugation, 1 ml of the ethyl acetate layer was counted in a liquid scintillation spectrometer.

Electrophoretic analysis -- Cellulose thin layer plates (Merck) were used for electrophoresis at 4° C, 17 v/cm potential gradient, for time periods noted in the figure legends. Buffers were prepared by titrating sodium formate with formic acid and diluting to a final sodium concentration of 0.2 M. The buffers were of various pH (given in figure legends) but all of ionic strength 0.2. Radioactivity was located on the plates by autoradiography on Kodak Royal Blue X-ray film. Non-radioactive methionine-containing compounds were located with the platonic iodide reagent described by Toennies and Kolb (1951). The position of the origin, corrected for endosmotic buffer flow, was located by (^{14}C)uridine.

Liquid scintillation counting -- Ethyl acetate aliquots (1 ml) were mixed with 10 ml Bray's solution (Bray, 1960) composed of 60 g naphthalene, 4 g 2,5-diphenyloxazole (New England Nuclear Corporation), 0.2 g 1,4-bis-[2-(5-phenyl-

oxazoly1)]-Benzene (Packard Instrument Company), 100 ml methanol, 20 ml ethylene glycol, and 834 ml dioxane.

Radioactivity was determined in a Nuclear Chicago liquid scintillation spectrometer at an efficiency of 50 to 60% (for ^{35}S).

RESULTS

E. coli ribosomal peptidyl transferase catalyzes a reaction in which the peptidyl donor is an N-formyl methionine containing hexanucleotide derived from formyl-methionyl-transfer RNA (CAACCA-fMet) and the acceptor is puromycin (Monro and Marcker, 1967). Thus when fMet-oligonucleotide fragment and puromycin are incubated in the presence of salt-washed ribosomes in a buffer containing potassium and magnesium ions and methanol, the product is N-formylmethionyl puromycin (Figure 1). This reaction requires no additional protein cofactors and no energy source (no GTP).

The formation of fMet-puromycin can be assayed by extraction with ethyl acetate from a pH 5.5 aqueous phase (Leder and Bursztyn, 1966). Derivatives of labelled methionine are extractable under these conditions only if they are uncharged, which requires that both the amino and carboxyl groups be blocked. When ψ hydroxypuromycin is incubated with ribosomes and a formyl (^3S)-methionine-labelled oligonucleotide fragment in such a system, an ethyl acetate-extractable product appears. The kinetics of the reaction are shown in Figure 2, for both puromycin and ψ hydroxypuromycin.

As is apparent from the "blank" in Figure 2, consider-

FIGURE 2 : TIME COURSE OF THE fMet-OLIGONUCLEOTIDE
FRAGMENT REACTION WITH ψ HYDROXYPUROMYCIN
AND PUROMYCIN.

Reaction conditions and assay were standard in the fragment system. The final concentration of puromycin was 2.5×10^{-4} M and the concentration of ψ hydroxypuromycin was 1.9×10^{-3} M. The "blank" sample was incubated without puromycin or ψ hydroxypuromycin. Each point represents a 0.1 ml aliquot which contained a total of 2730 dpm of (^{35}S) fMet oligonucleotide fragment. The ordinate value is $([\text{dpm in 1 ml ethyl acetate aliquot}] \times 150)/2730$.

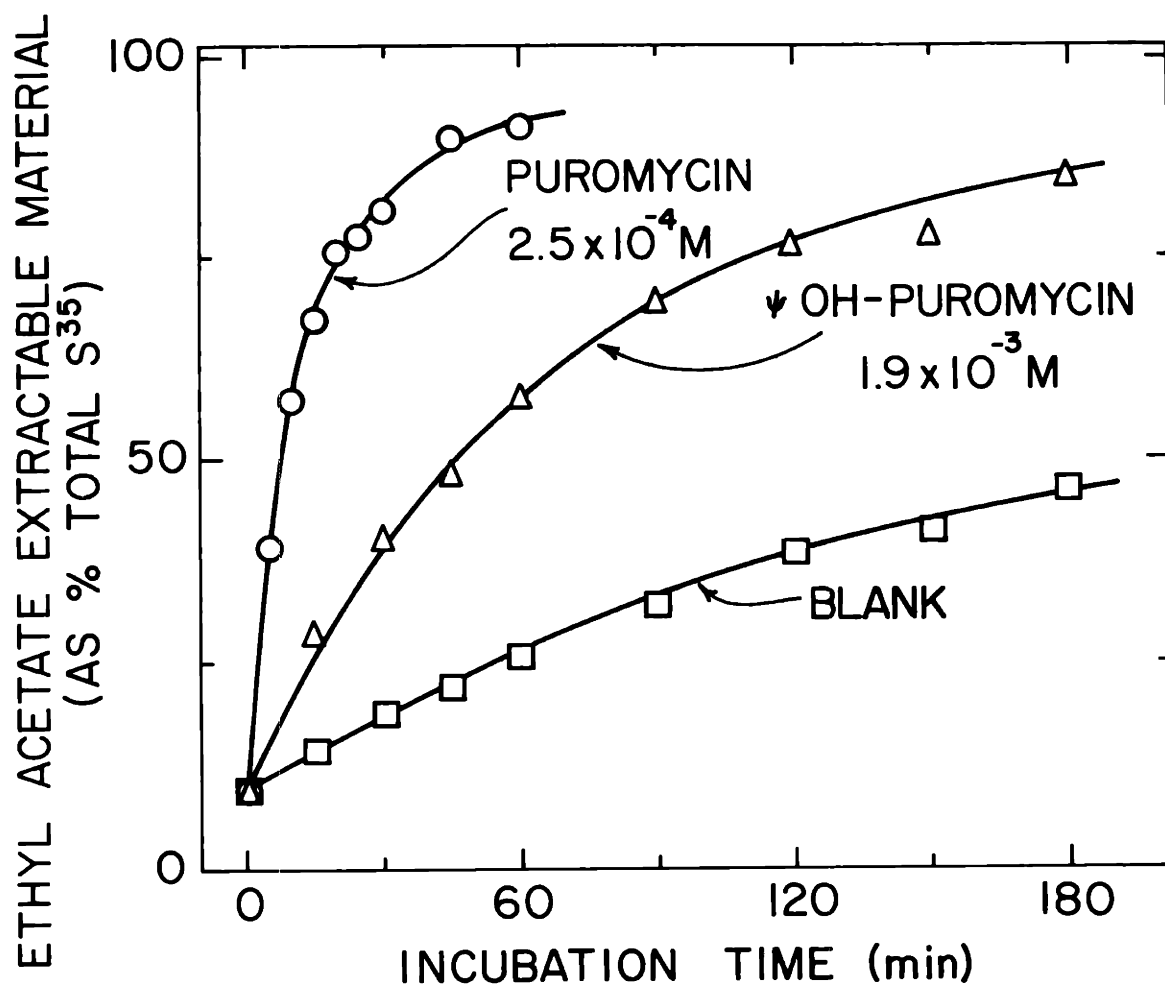


Figure 2

ethyl acetate-extractable material is formed in the absence of puromycin or ψ hydroxypuromycin. This material is formylmethionine methylester which is formed by a non-enzymatic methanolysis of the fMet fragment. Its identity was established by chromatographic and electrophoretic comparison to a standard formed by chemical formylation of methionine methylester. The formation of this ester is independent of ribosomes but dependent on the pH and the concentration of methanol. It is exaggerated by the relatively high pH (8.1) and concentration of methanol (50% v/v) used in these experiments. The reactions are terminated with BeCl_2 which is slightly acidic and lowers the pH sufficiently to stop the nonenzymatic methanolysis as well as the enzymatic reactions. The methylester formation is quite unrelated to the enzymatic reactions of interest here.

As shown in Table 1, the stimulation due to ψ hydroxypuromycin is dependent upon ribosomes and is not observed when post-ribosomal supernatant is substituted for ribosomes. The reaction requires the presence of magnesium and potassium ions and is inhibited by two inhibitors of ribosomal peptidyl transferase, chloramphenicol and gougerotin. In these respects the reaction is identical to the puromycin reaction.

Although chloramphenicol and gougerotin both inhibit the action of peptidyl transferase (Monro and Vazquez, 1967).

TABLE 1
REACTION BETWEEN THE fMet-OLIGONUCLEOTIDE FRAGMENT
AND ψ HYDROXYPUROMYCIN

	ψ HYDROXY- PUROMYCIN (1.0×10^{-3} M)	PUROMYCIN (6.9×10^{-5} M)
Complete system	350 dpm	827 dpm
Minus Mg^{2+}	28 dpm	16 dpm
Minus K^{+}	28 dpm	-3 dpm
Minus ribosomes	33 dpm	15 dpm
Minus ribosomes plus post- ribosomal supernatant (15 μ g protein)	20 dpm	-37 dpm
Plus 1 mM chloramphenicol	-39 dpm	-15 dpm
Plus 1 mM gougerotin	45 dpm	5 dpm

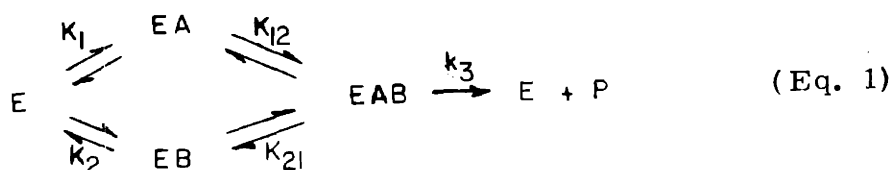
The complete system was as described in Methods. Aliquots (0.2 ml) containing 5400 dpm of (^{35}S) fMet-fragment were incubated for 15 min. then assayed by ethyl acetate extraction. Blanks (without ψ hydroxypuromycin or puromycin) were incubated along with samples containing 1.0×10^{-3} M ψ hydroxypuromycin or 6.9×10^{-5} M puromycin. The values listed are dpm in the ethyl acetate aliquot after subtraction of the appropriate blank value. The blank values ranged from 304 dpm to 366 dpm, except the sample with gougerotin, which was 230 dpm. Values reflect initial reaction rates.

their modes of action are probably somewhat different. Chloramphenicol binds to a single specific site on the 50S subunit (Vazquez, 1964), presumably the site at which its inhibitory effect on peptidyl transferase is exerted (Vazquez, 1966). Gougerotin does not inhibit the binding of chloramphenicol to ribosomes (Vazquez and Monro, 1967), therefore it presumably acts at a different site. Thus, these two inhibitors are not simply interchangeable and the inhibition of the ψ hydroxypuromycin reaction by both of them indicates that it is sensitive to inhibition at both sites, as is the puromycin reaction.

The value of the blank (fMet-methylester formation) in Table 1 is not dependent on ribosomes, etc., but it is inhibited somewhat by gougerotin. This effect is probably related to the fact, which has been reported by Celma, et al (1970), that gougerotin stimulates the binding of an N-acetyl leucine pentanucleotide fragment to the ribosome. A similar effect is likely to occur with the fMet-fragment. On the ribosome, the fragment is probably protected from attack by methanol, just as aminoacyl-tRNA has been shown to be protected from alkaline hydrolysis when bound to a ribosome (Pestka, 1967). This interpretation is supported by the fact that chloramphenicol, which also inhibits peptidyl transferase but which has a much smaller stimulatory effect on ribosome binding of the

fragment, does not lower the blank value.

The dependence of the rate of this reaction (i.e., the rate of formation of ethyl acetate extractable material in excess of that formed in the blank) on the concentration of ψ hydroxypuromycin is shown in Figure 3. This Lineweaver-Burk plot appears to give a straight line for both the puromycin and ψ hydroxypuromycin data. The simplest interpretation of this behavior is a simple Michaelis-Menten Model,



where A and B are puromycin (or ψ hydroxypuromycin) and the fMet-fragment respectively, K_1 , K_2 , etc. are dissociation constants, and k_3 , a rate constant, is slow compared to the establishment of the enzyme-substrate equilibria. Let us assume that the dissociation constant of each substrate is independent of the binding of the other (i.e., that $K_1 = K_{21} = K_A$ and $K_2 = K_{12} = K_B$). Then, as shown by Webb (1963), the rate law can be written

$$\frac{1}{v} = \frac{1}{\bar{V}_{\max}} \left(1 + \frac{K_A}{A}\right) \left(1 + \frac{K_B}{B}\right) \quad (\text{Eq. 2})$$

where v is the observed reaction velocity, \bar{V}_{\max} the maximal velocity with both substrates saturating, and A and B the substrate concentrations.

According to this model reciprocal plots of $1/v$ vs.

FIGURE 3 : DEPENDENCE OF INITIAL REACTION RATE UPON THE CONCENTRATION OF ψ HYDROXYPUROMYCIN AND PUROMYCIN.

Reaction conditions and assay were standard in the fragment system. Aliquots (0.2 ml) containing puromycin were incubated 3.5 min., those containing ψ hydroxypuromycin were incubated 15 min. Each aliquot contained 2670 cpm of formylmethionyl oligonucleotide fragment. A blank was also incubated for each of these times without puromycin or ψ hydroxypuromycin. The value obtained for the appropriate blank was subtracted from that of each sample. The data are presented as Lineweaver-Burk plots in which the units of the ordinate are reciprocal of cpm in the ethyl acetate aliquot minus blank and the units of the abscissa are reciprocal molar concentration of puromycin or ψ hydroxypuromycin. (a) Data for ψ hydroxypuromycin. (b) Data for puromycin.

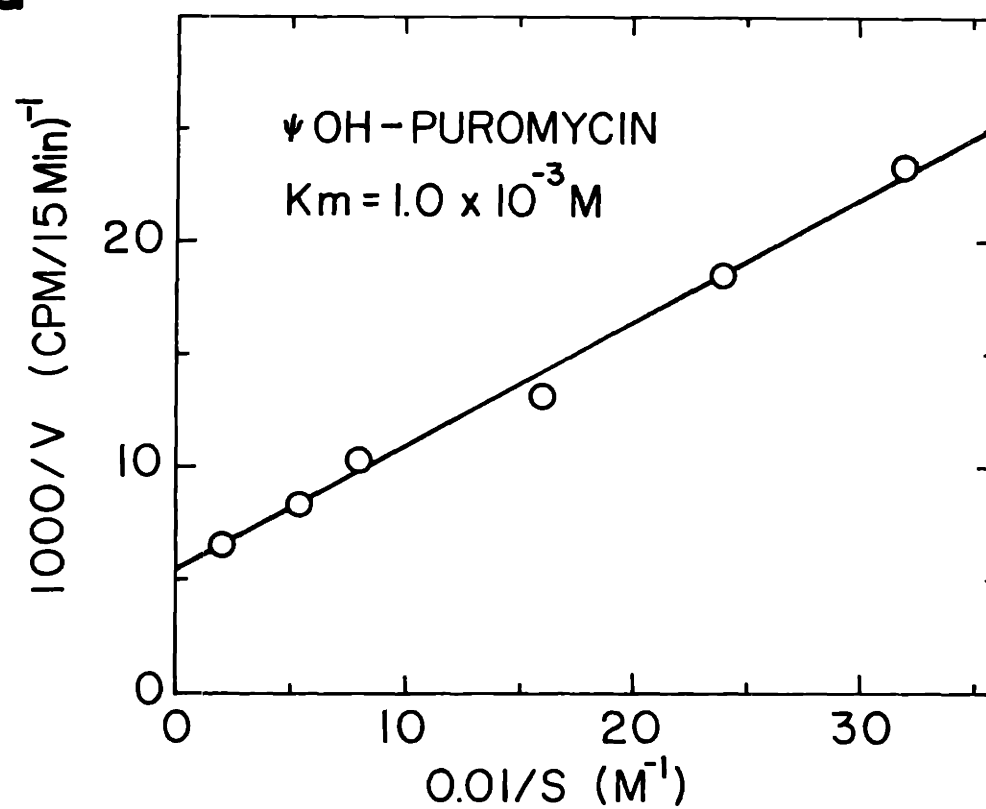
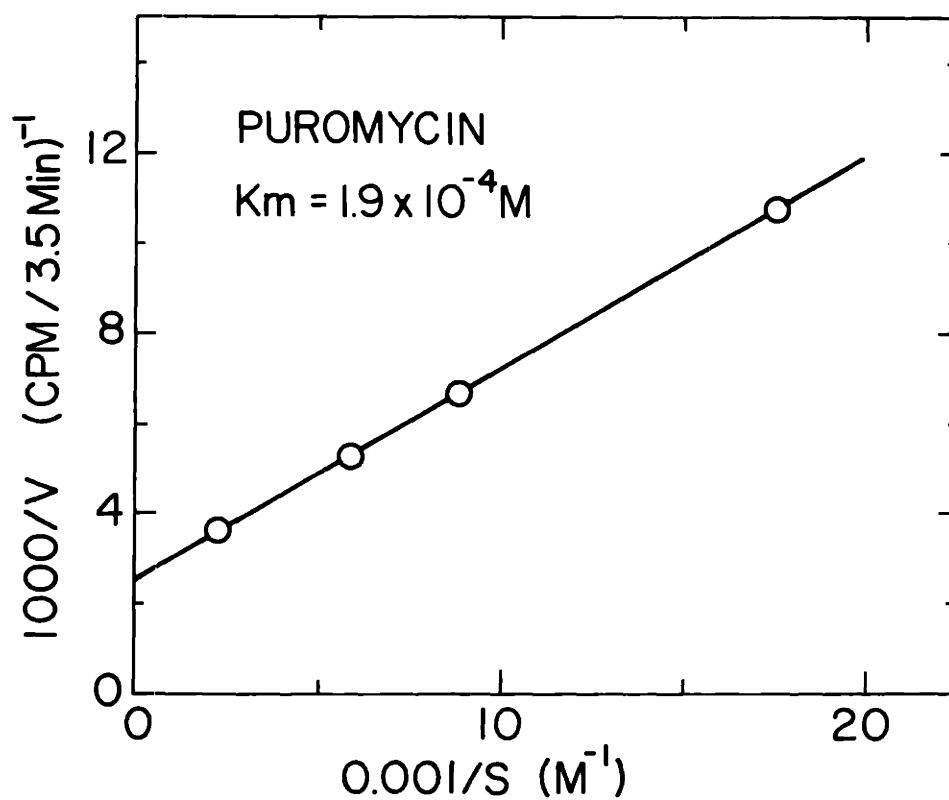
a**b**

Figure 3

$1/(\text{Puromycin})$ at various concentrations of the fragment should intersect on the abscissa at $-1/K_A$. Reciprocal plots for puromycin do intersect on the abscissa, as shown in Figure 4. Since, as predicted by the simple model, the observed K_m for puromycin is independent of the concentration of the fragment, it is reasonable to assume that the K_m derived from a single reciprocal plot at any concentration of the fragment is the substrate dissociation constant.

However, in such plots the intercept on the $(1/v)$ axis is $(1/V_{\max})(1 + K_B/B)$. Thus V_{\max} cannot be determined directly from such a plot. Instead it can be assumed, since K_B (the dissociation constant of the fMet-fragment) is independent of the binding of puromycin, that the value of K_B is the same for reaction with puromycin or its analogs. Thus values of V_{\max} for puromycin analogs are expressed as a ratio to the V_{\max} for puromycin measured at the same time under the same conditions. Then these ratios for different compounds measured in different experiments can be compared since the factor $(1 + K_B/B)$ is cancelled out of the ratio.

Of course one must exercise caution in interpreting kinetic data on an enzyme system as complex as a ribosome. Nevertheless, in the fragment system it is likely, in view of the relatively simple structures of the substrates and

FIGURE 4 : DEPENDENCE OF KINETIC PARAMETERS FOR
PUROMYCIN ON THE CONCENTRATION OF
fMet-OLIGONUCLEOTIDE FRAGMENT.

Reaction and assay conditions were standard in the fragment system. Aliquots (0.2 ml) containing various concentrations of puromycin were incubated for 5 min. along with blanks lacking puromycin. The appropriate blank value was subtracted from each. Each aliquot contained the following amount of fMet-Oligonucleotide fragment: I, 1839 cpm; II, 3794 cpm; III, 7680 cpm. The data are presented as Lineweaver-Burk plots in which the units of the ordinate are reciprocal of cpm in the ethyl acetate aliquot minus blank and the units of the abscissa are reciprocal molar concentration of puromycin.

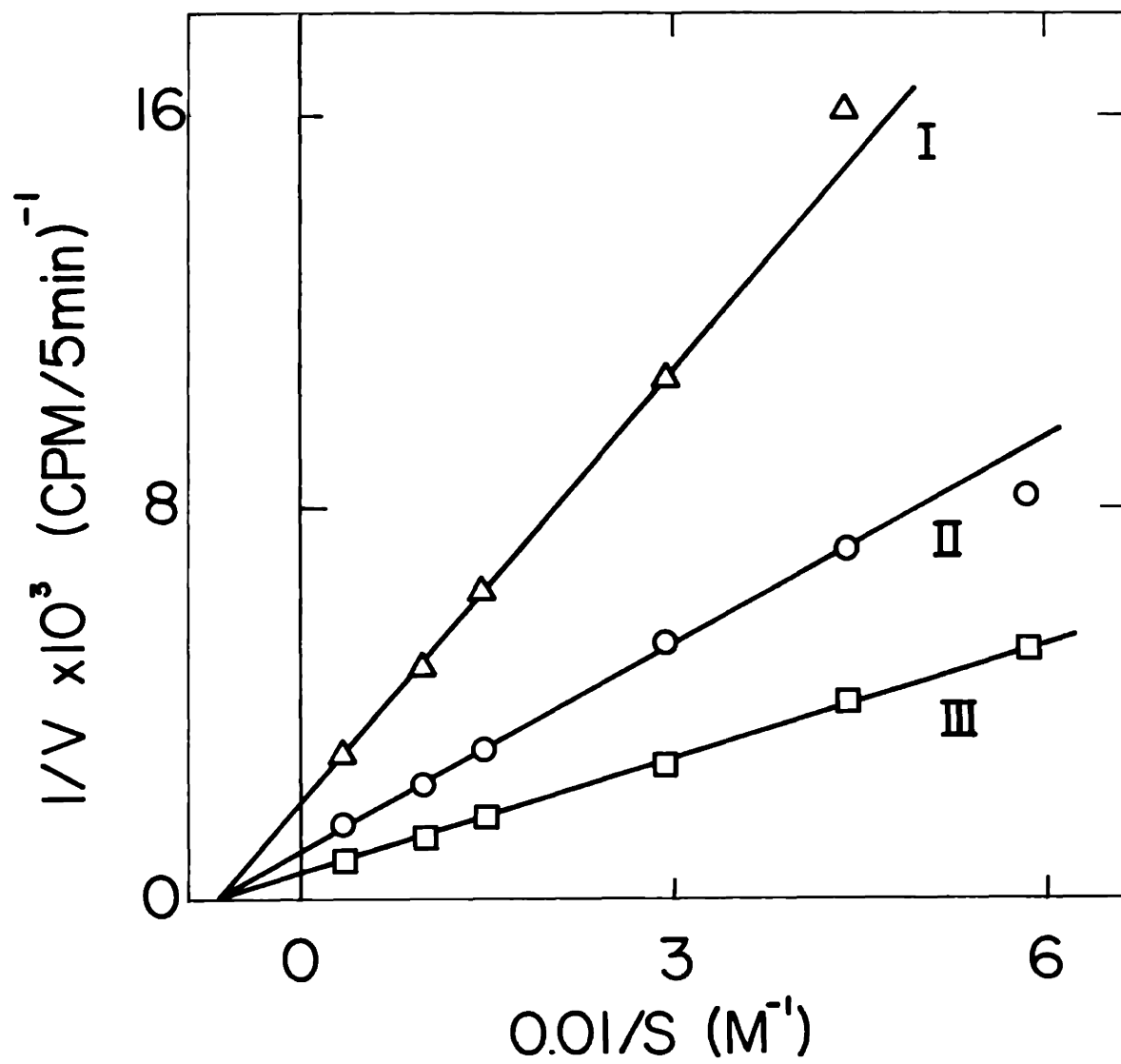


Figure 4

the absence of protein factor and energy requirement, that the reaction involves only the peptidyl transferase active site. In that case the simple model described above is a reasonable working hypothesis.

The Michaelis constant, K_m , for ψ hydroxypuromycin in Figure 3 is 1.0×10^{-3} M. The K_m for puromycin under the same conditions is 1.9×10^{-4} M. The ratio of the maximal reaction velocity (V_{max}) for ψ hydroxypuromycin to that for puromycin is 0.11. Thus substitution of a hydroxyl group for the α amino group of puromycin (and elimination of the p-methoxy group) decreases the binding affinity by a factor of 5 and decreases the maximal reaction velocity by a factor of 9 (assuming the simplest interpretation of these constants).

ψ hydroxypuromycin is synthesized from the L isomer of β -phenyllactic acid. However, if racemic D,L- phenyllactic acid is used, the resulting compound has a K_m twice that of the L-isomer (2.2×10^{-3} M), as shown in Figure 5. Its V_{max} is roughly the same as that of the L-isomer (0.09 times the V_{max} for puromycin, compared to 0.11 for the L-isomer). This suggests that in the mixture only the compound derived from the L-isomer of phenyllactic acid is active. Similarly an analog of puromycin derived from the D amino acid is inactive (Nathans and Niedle, 1963). Therefore, the reaction of ψ hydroxypuromycin resembles

FIGURE 5 : DEPENDENCE OF INITIAL REACTION RATE
UPON CONCENTRATION OF DL- ψ -HYDROXY-
PUROMYCIN.

Reaction and assay conditions were as described in Methods. Aliquots (0.2 ml), each containing 2045 cpm of formylmethionyl-T₁ fragment, were incubated for 30 min. along with a blank lacking DL- ψ -hydroxypuromycin. The value of the blank was subtracted from that of each sample. The data are presented as a Lineweaver-Burk plot in which the units of the ordinate are reciprocal of cpm in the ethyl acetate aliquot minus blank, and the units of the abscissa are reciprocal molar concentration of DL- ψ -hydroxypuromycin.

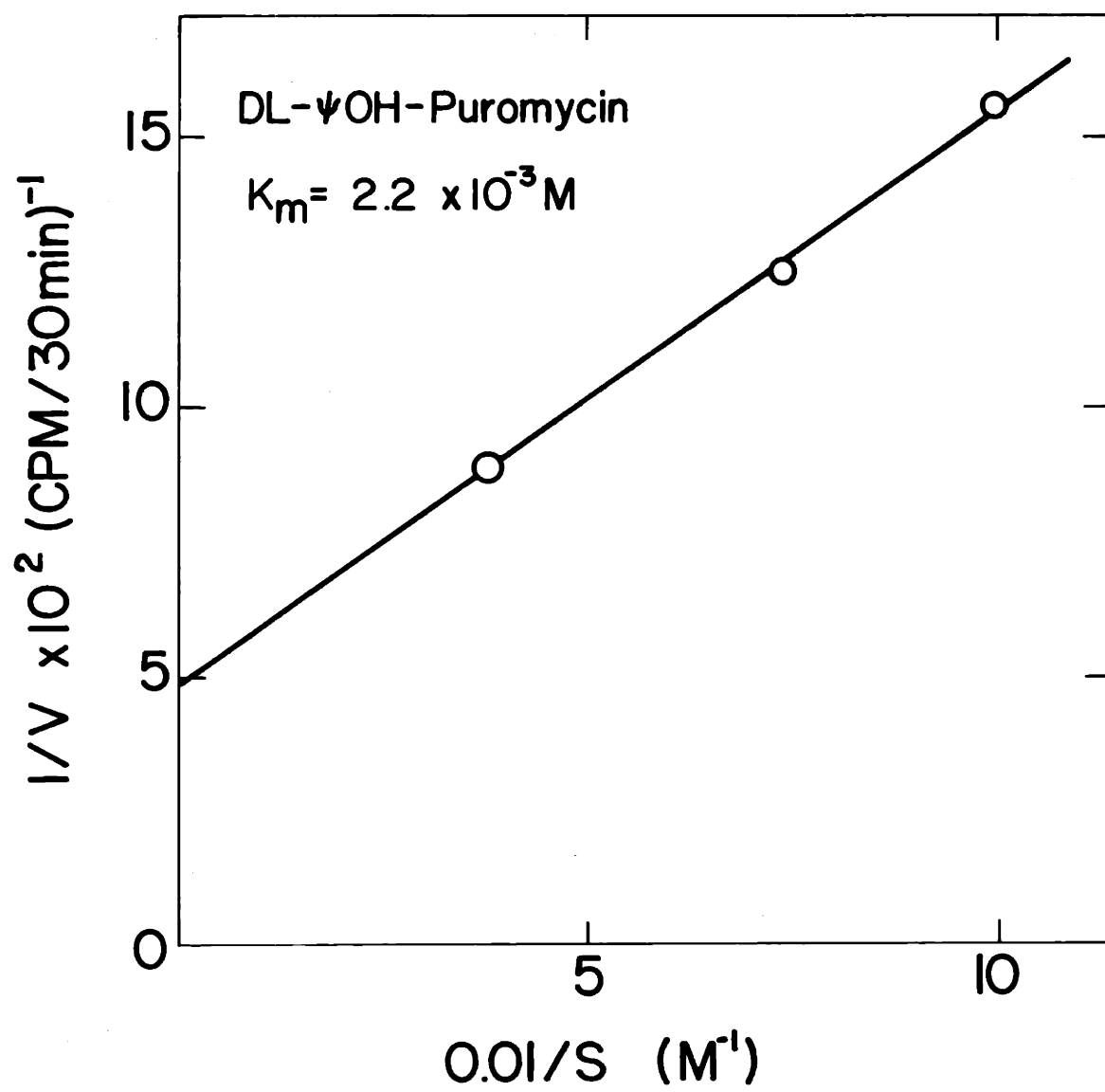


Figure 5

the puromycin reaction in this respect also.

Characterization of the product

Figure 6 shows the electrophoretic behavior of the products synthesized in the fMet-fragment reaction. Panels (a) through (d) show the mobilities, at several values of pH, of the ethyl acetate extractable radioactive products formed in the presence of puromycin (P) or ψ hydroxy-puromycin (O), and the blank (B). The fMet methylester formed in the blank is uncharged throughout this pH range and remains at the origin, which is marked by (^{14}C)uridine (U). The origin located in this way is not the point at which the material was initially spotted, but rather the point at which uncharged compounds are located. This is due to the flow of solvent over the plate in the direction of the cathode, the endosmotic effect.

The product formed in the presence of puromycin is fMet-puromycin (Figure 1). The presence of ψ hydroxy-puromycin results in the appearance of a spot (arrows) which has a mobility very similar to that of fMet-puromycin at each pH. In the range between pH 3.0 and 4.2 all charge on fMet-puromycin is due to protonation of the purine dimethylamino group which has a pK_a of 3.7 (Nathans, 1967). As seen in Figure 6 the mobility of fMet puromycin increases between pH 4.2 and pH 3.0 as the dimethylamino group assumes a higher charge. The ψ hydroxypuromycin product has the

FIGURE 6 : ELECTROPHORETIC COMPARISON OF fMet-
PUROMYCIN AND fMet- ψ -HYDROXYPUROMYCIN.

Products were formed from (^{35}S) fMet T₁ oligonucleotide under conditions as described under Methods, except that the ribosome concentration was 23 A₂₆₀ units/ml and the fMet-oligonucleotide radioactivity was 3.8×10^5 dpm/ml prior to methanol addition. Samples labelled P contained a final concentration of 5×10^{-4} M puromycin, those labelled O contained 5×10^{-4} M ψ hydroxypuromycin, and those labelled B (blank) contained no puromycin or ψ hydroxypuromycin. Samples were incubated 2 1/2 hours at 0° C, then the reaction was terminated and the samples extracted with ethyl acetate as described under Methods. The ethyl acetate layer was dried under reduced pressure and the residue was dissolved in ethanol. Aliquots of each of these solutions were incubated in 1 M triethylamine for 30 min. at 37° C, dried under reduced pressure, and the residue redissolved in ethanol. These triethylamine-treated samples are designated in (e) by P', O', and B', respectively.

Aliquots of the products were subjected to electrophoresis as described under Methods. The direction of the cathode is up in all cases. The material labelled U is (^{14}C)uridine used to locate the origin, corrected for the endosmotic effect. Arrows indicate the position of fMet- ψ hydroxypuromycin.

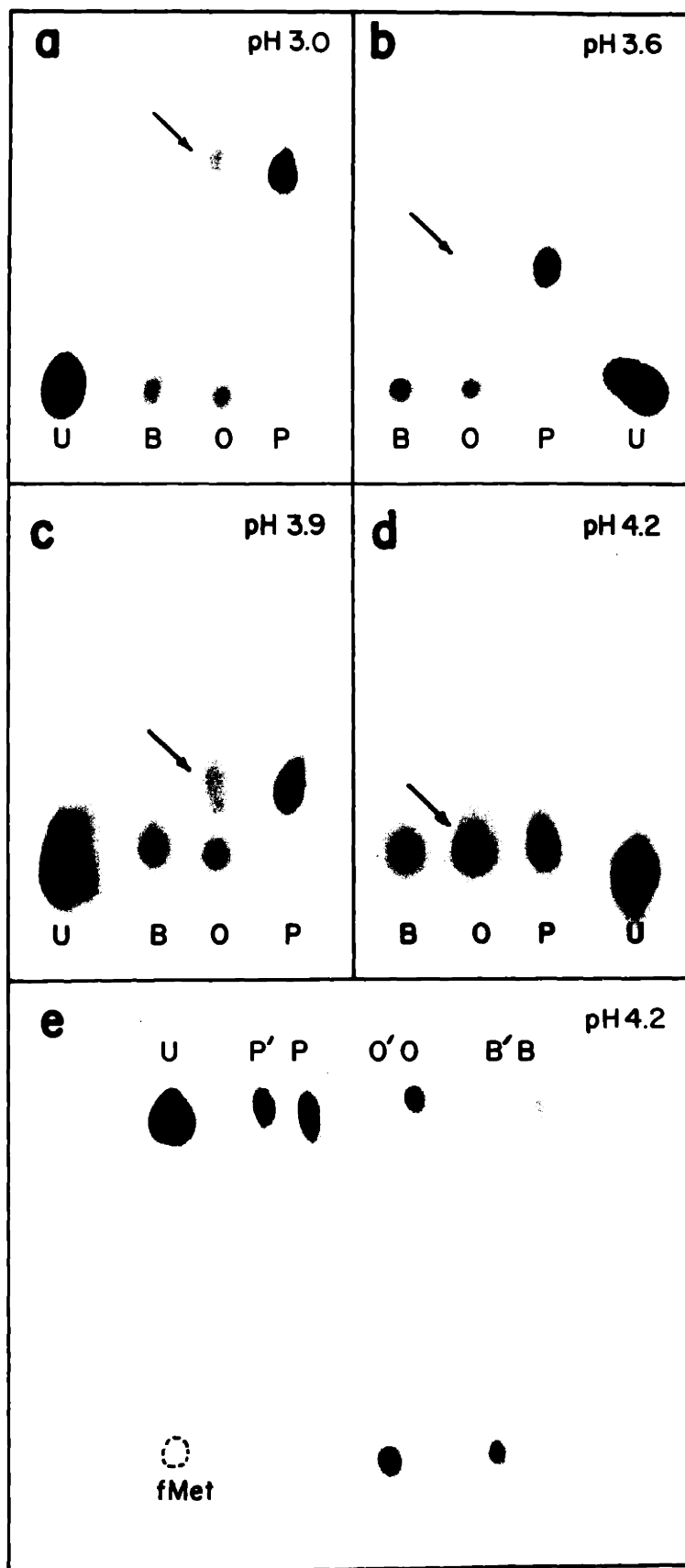


Figure 6

same properties and this suggests that it also contains the nucleoside moiety of ψ hydroxypuromycin.

In Figure 6 (e) the samples labelled with primes (P', O', B') were treated with 1 M triethylamine (pH 12.5) prior to electrophoresis. The product formed in the presence of ψ hydroxypuromycin (fMet- ψ hydroxypuromycin) is destroyed by this treatment with release of N-formylmethionine. This behavior is indicative of an ester bond between fMet and ψ hydroxypuromycin. In this respect fMet- ψ hydroxypuromycin differs from fMet-puromycin, which is not affected by this alkaline treatment because the bond between fMet and puromycin is an amide. The fMet methylester in the blank (B) is also hydrolyzed to fMet.

fMet- ψ hydroxypuromycin is also hydrolyzed under relatively mild alkaline conditions as shown in Figure 7. While fMet-puromycin is unaffected at pH 9.0, fMet- ψ hydroxypuromycin begins to be hydrolyzed appreciably at a pH just over 8 and is destroyed rapidly at pH 9.0. The methylester (blank product) is also hydrolyzed, but more slowly than the ψ hydroxypuromycin product (Figure 7 [b]). Thus, the rapidly hydrolyzed material is probably the fMet- ψ hydroxypuromycin, though this was not demonstrated here. It will be made clear below by comparison with a similar product formed in an aqueous system where the methylester is not

FIGURE 7 : ALKALINE HYDROLYSIS OF PRODUCTS
FORMED IN THE FRAGMENT SYSTEM.

Products formed from (^{35}S) fMet- T_1 oligonucleotide fragment under standard conditions in the presence of puromycin (2×10^{-4} M) or ψ hydroxypuromycin (1.1×10^{-3} M) or in the absence of both compounds (blank) were extracted with ethyl acetate in the usual manner. The solutions were dried under reduced pressure and the material redissolved in ethanol. Aliquots of these ethanol solutions were mixed with 0.04 M Tris-HCl buffer at the pH indicated and incubated at 30°C . (Final ethanol concentration 1.5% v/v) Aliquots were removed at various times for assay by ethyl acetate extraction.

- (a) Filled circles: puromycin product.
Open symbols: ψ hydroxypuromycin product.
(b) Blank product (fMet-methylester).

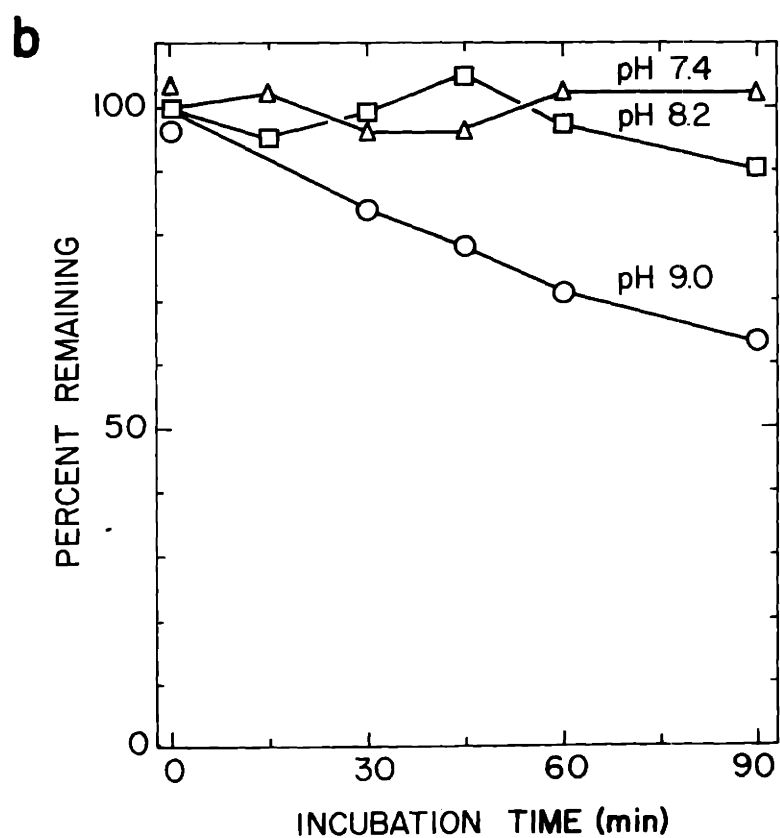
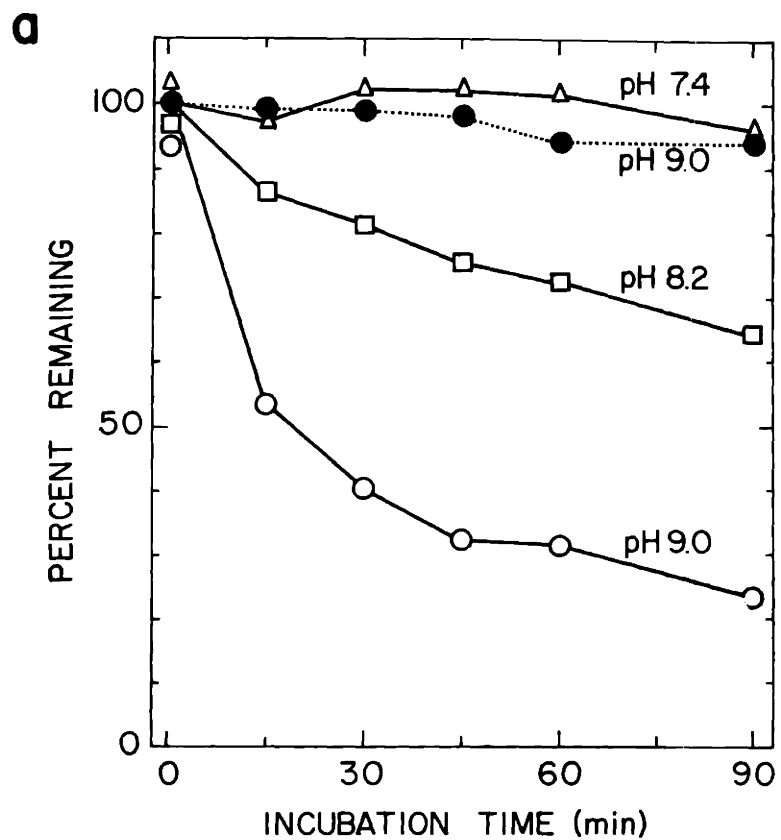


Figure 7

present.

The nature of the catalytic unit

The properties shown in Table 1 suggest that the reaction of ψ hydroxypuromycin is catalyzed by the same enzyme which catalyzes the puromycin reaction, i.e., ribosomal peptidyl transferase. Further similarity of these reactions is shown by the heat inactivation data in Figure 8. Ribosomes were preincubated for 5 minutes at various temperatures then tested for their activity in a subsequent incubation in the fragment system at 0° C. Preincubation of the ribosomes at temperatures above 60° C results in a parallel loss of their ability to catalyze fMet-puromycin and fMet- ψ hydroxypuromycin formation. A similar heat inactivation profile has been observed for a number of ribosomal functions (McLaughlin, et al, 1968; Bodley, 1969). Thus, it is likely that this inactivation reflects an overall destruction of ribosome structure at high temperatures. In that case the results shown in Figure 8 imply that both the puromycin reaction and the ψ hydroxypuromycin reaction are catalyzed by an enzyme which depends for its activity on the structural integrity of the ribosome. Thus, at least it is unlikely that the ψ hydroxypuromycin reaction is catalyzed by a nonribosomal contaminant in the ribosome preparation, in agreement with the conclusion derived from the data in Table 1.

FIGURE 8 : HEAT INACTIVATION OF RIBOSOMES.

Ribosomes (13.8 A_{260} units per ml) were incubated in 0.4 M KCl, 0.02 M magnesium acetate, 0.06 M Tris-HCl (pH 8.1 at 0° C) for 5 min. at various temperatures, then kept at 0° C for 15 min. (35 S) fMet T_1 oligonucleotide (50,000 dpm/ml) was added and the mixture divided into 0.1 ml aliquots. To each aliquot was added 0.1 ml methanol (blank) or a methanol solution of ψ hydroxypuromycin (2.0×10^{-3} M) or puromycin (1.0×10^{-4} M). After 15 min. at 0° C the reaction was terminated and product assayed as described in Methods.

The values obtained reflected initial rates of the reaction. The data are expressed as percentage of the value obtained using ribosomes kept at 0° C throughout (control) after subtraction of the appropriate blank. The control values were 644 dpm for puromycin and 174 dpm for ψ hydroxypuromycin.

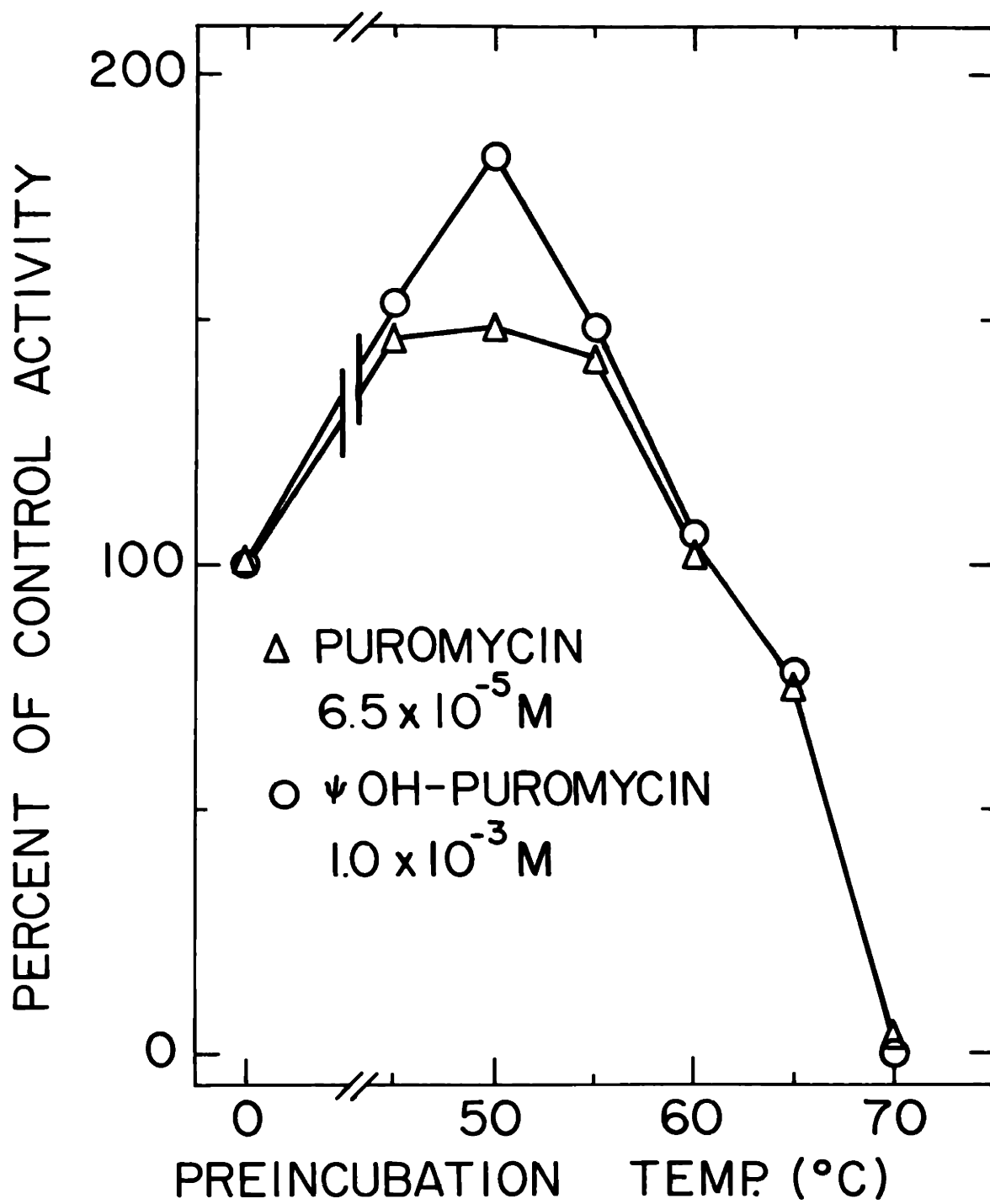


Figure 8

At temperatures below 60° C an activation of the ribosomes is observed. Similar activation of ribosomes has been reported by others (Bodley, 1969; Miskin, et al, 1968). Though the inactivation at temperatures above 60° C in Figure 8 is parallel for the two reactions the ψ hydroxypuromycin reaction is relatively more activated below 60° C than is the puromycin reaction. The inference to be derived from this (reproducible) difference is not clear. In the case of thermal denaturation the phenomenon is most likely an all or none inactivation as far as an individual enzyme molecule is concerned, so all reactions catalyzed by a given enzyme are likely to be inactivated in parallel. The nature of the activation may be a bit more subtle. If it represented an all or none activation of previously inactive ribosomes one might expect that the activation of the two reactions would be parallel. However, if the activation is an increase in the activity of already active ribosomes it could easily give rise to greater relative activation of one reaction or the other. For example, the effect might be due to some rearrangement of the structure of the ribosome which affects the geometry of the enzymatic active site. This rearrangement might be reflected in an increase of the affinity of the enzyme for the substrates, so that the observed degree of activation will depend upon the substrate concentration.

Alternatively of course, this difference may be taken as evidence that the two reactions are catalyzed by different subsets of the ribosome population. This interpretation is unlikely in view of evidence to be presented below, and the preceding argument is intended only to show that it is not an unavoidable interpretation.

As discussed above, the observed K_m for an acceptor substrate can be interpreted on the basis of the simplest model to represent the dissociation constant of the enzyme substrate complex. In this way the binding constant of each substrate to sites which catalyze its reaction can be determined. It is also possible to determine the binding constant of ψ hydroxypuromycin to sites which catalyze the puromycin reaction. In Figure 9 the rate of formation of fMet-puromycin is measured specifically in the presence of ψ hydroxypuromycin by modifying the assay to include alkaline treatment which destroys fMet- ψ hydroxypuromycin. The consumption of fMet by reaction with ψ hydroxypuromycin does not interfere with the measurement of the puromycin reaction since the measurement involves initial rates of reaction, before substrate depletion becomes important. The data are presented as Lineweaver-Burk plots in Figure 9. The presence of ψ hydroxypuromycin results in inhibition of the puromycin reaction. The data in the presence and absence of

FIGURE 9 : INHIBITION OF THE PUROMYCIN REACTION
BY ψ HYDROXYPUROMYCIN.

Reaction conditions were standard in the fragment system. Aliquots (0.2 ml) containing 11,175 cpm of fMet fragment and various concentrations of puromycin only (O) or various concentrations of puromycin plus 2×10^{-3} M ψ hydroxypuromycin (Δ), and blanks lacking puromycin were incubated for 3 min. The reaction was terminated with 0.025 ml 0.1 M BeCl_2 . To each sample was added 0.2 ml 1 N KOH. After incubation for 15 min. at 0°C the samples were neutralized with 0.2 ml 1 N HClO_4 . Brief centrifugation pelleted the KClO_4 precipitate. An aliquot (0.3 ml) of the supernatant was mixed with 0.5 ml 0.3 N sodium acetate, pH 5.5, saturated with magnesium sulfate, and extracted with 1.5 ml ethyl acetate. One ml of the ethyl acetate layer was counted in a liquid scintillation spectrometer. The data are presented as Lineweaver-Burk plots in which the units of the ordinate are reciprocal of cpm in the ethyl acetate aliquot minus blank and the units of the abscissa are reciprocal molar concentration of puromycin.

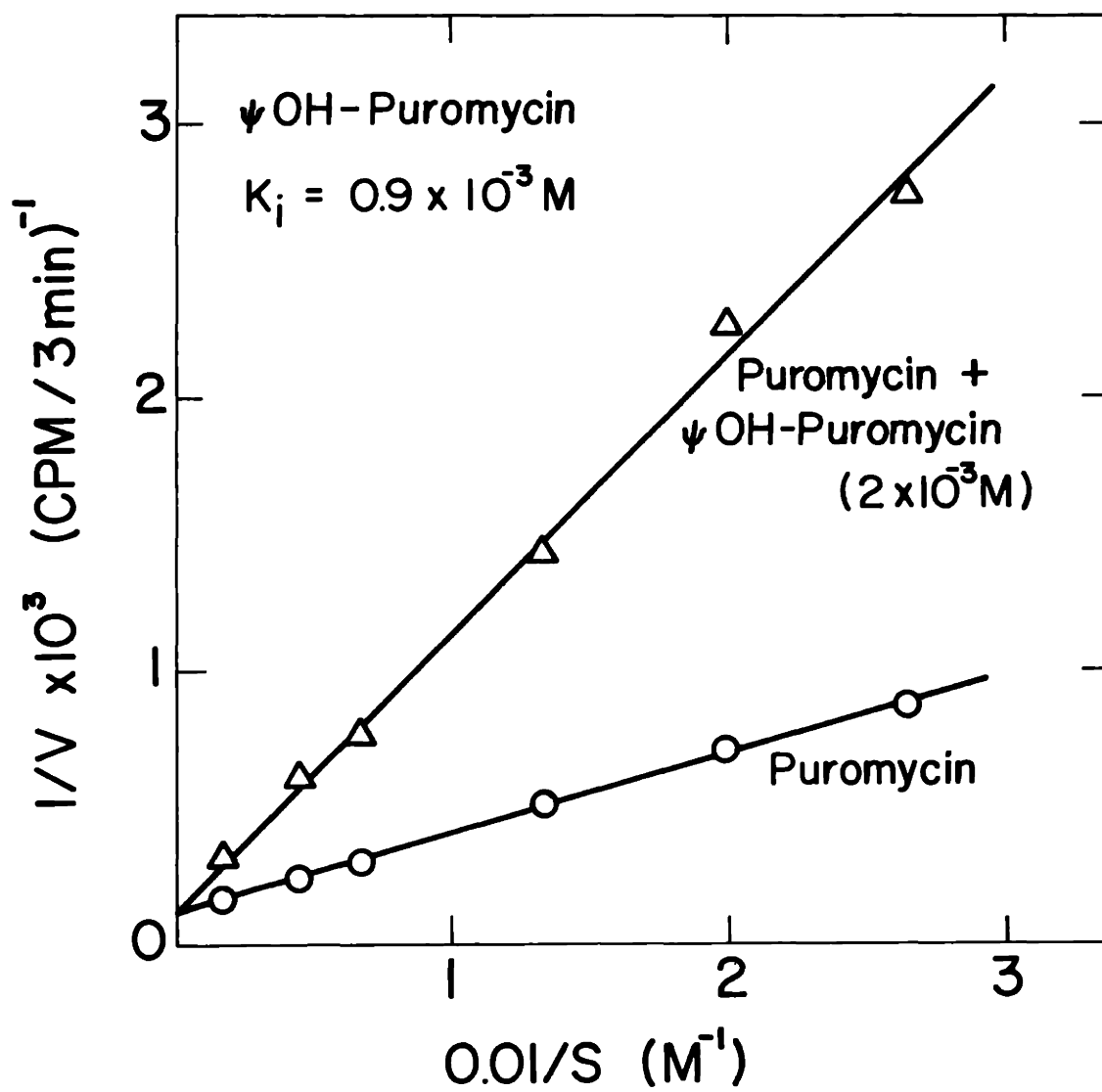


Figure 9

ψ hydroxypuromycin appear to fit straight lines which intersect at the ordinate. Thus the inhibition by ψ hydroxypuromycin appears to be competitive with puromycin. This competitive inhibition results from binding of ψ hydroxypuromycin to enzymatic sites which catalyze the puromycin reaction, preventing the binding of puromycin.

Assuming that ψ hydroxypuromycin (I) inhibits the puromycin reaction by competitively preventing the binding of puromycin to the enzyme and assuming the model described in Eq. 1, then, as shown by Webb (1963), the rate equation can be written

$$\frac{1}{v} = \frac{1}{V_{\max}} \left(1 + \frac{K_B}{B} \right) \left\{ 1 + \left[\frac{K_A}{A} \left(1 + \frac{I}{K_i} \right) \right] \right\} \quad (\text{Eq. 3})$$

where I is the inhibitor concentration and K_i is its dissociation constant. Thus in the presence of inhibitor the (1/S) intercept of a reciprocal plot (1/v vs 1/Puromycin) is $1/K_A \left(\frac{1}{1 + I/K_i} \right)$. Measuring this intercept in the presence and absence of inhibitor allows calculation of the dissociation constant of the enzyme inhibitor complex, K_i . It should be reemphasized that the interpretation of all of these constants is based on the assumption of the simplest possible kinetic model.

The dissociation constant, K_i , determined from the effect of ψ hydroxypuromycin on the apparent K_m for puromycin, is 0.9×10^{-3} M. The dissociation constant,

K_m , for hydroxypuromycin measured in its reaction is 1.0×10^{-3} M. These two numbers are the same, within the accuracy with which K_i is determined. If these constants can be interpreted in the simplest way, then the binding constant of ψ hydroxypuromycin to sites which catalyze its reaction is the same as its binding constant to sites which catalyze the puromycin reaction. Therefore either the same sites catalyze both reactions or the two kinds of sites are very similar.

Maden and Monro (1968) have shown that the activity of ribosomal peptidyl transferase increases with increasing pH between 7.0 and 8.5. A similar pH dependence is observed for the reaction of ψ hydroxypuromycin, as shown in Figure 10. In this experiment the initial reaction velocity was measured for both puromycin and ψ hydroxypuromycin in buffers of various pH values. The pH was measured at 0° C in a similar mixture without ribosomes and the fMet fragment and prior to methanol addition. Thus the pH listed is not exactly the pH at which the reaction occurred, but may be several tenths of a unit different.

Maden and Monro noted that this pH dependence suggests that a functional group with a pK_a value in the range 7.5 to 8.0 is involved in the catalysis. However, as those authors suggested, the pH dependence for the puromycin reaction might alternatively be due to proto-

FIGURE 10: EFFECT OF pH ON THE RATE OF REACTION
BETWEEN fMet OLIGONUCLEOTIDE AND
 ψ HYDROXYPUROMYCIN OR PUROMYCIN.

Prior to addition of methanol, 0.1 ml reaction mixtures contained: ribosomes (1.38 A_{260} units); fMet- T_1 oligonucleotide (5,900 dpm); 0.4 M KCl; 0.02 M magnesium acetate; 0.06 M N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonate buffer. The pH was determined at 0° C in a similar mixture without ribosomes and the fMet oligonucleotide. The reaction was initiated by adding 0.1 ml methanol (blank) or a methanolic solution of ψ hydroxypuromycin (2.0×10^{-3} M) or puromycin (1.1×10^{-4} M). After 15 min. at 0° C, the reaction was terminated and assayed as described under Methods, except that 1 ml of 0.3 M sodium acetate, pH 5.5, was used in the extraction. Blank values (from 48 dpm at pH 7.2 to 254 dpm at pH 8.5) were subtracted. Data are expressed as percent of the pH 8.5 values, which were 368 dpm for puromycin and 175 dpm for ψ hydroxypuromycin.

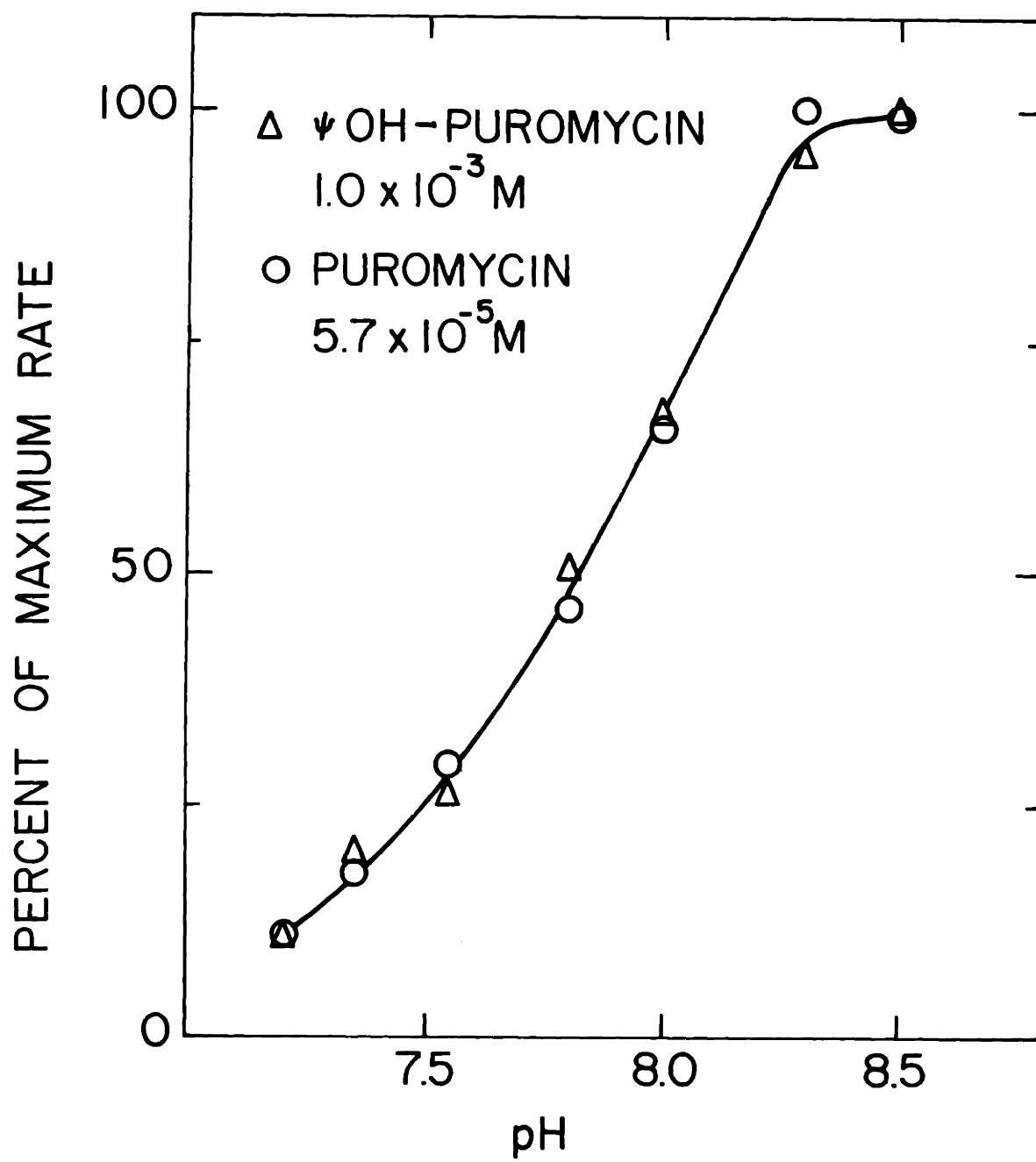


Figure 10

nation of the α -amino group of puromycin if its pK is altered slightly on complex formation (It is 7.3 in aqueous solution [Nathans, 1967]). This ambiguity is eliminated by the present observation, because the similar pH dependence of the ψ hydroxypuromycin reaction clearly cannot be explained by substrate protonation.

This is consistent with the proposal that an imidazole residue or N-terminal α -amino group might be involved in the catalysis of the reaction. The pH in this experiment probably affects peptidyl transferase specifically and not, for example, by causing gross conformational changes in the ribosome. The pH dependence observed here is stronger than the dependence of protein synthesis viewed in its entirety (Matthaei and Nirenberg, 1961), and there is no such pH dependence for other ribosomal processes such as chloramphenicol binding (Maden and Monro, 1968) and messenger RNA binding (Dahlberg and Haselkorn, 1967).

The concentration dependence of the rate of the puromycin reaction was determined at two different values of pH in order to determine which of the kinetic parameters is affected by pH changes. These data, as Lineweaver-Burk plots, are shown in Figure 11. It is the apparent V_{\max} which is affected most strongly by the difference in pH, while the apparent K_m is affected very little if at all. Thus, again assuming that these parameters can

FIGURE 11: pH DEPENDENCE OF THE KINETIC PARAMETERS
FOR PUROMYCIN IN THE fMet FRAGMENT
SYSTEM.

Reaction and assay conditions were standard in the fragment system, except that prior to methanol addition the pH was either 7.4 (Δ) or 8.1 (o) (measured at 0° C). Aliquots (0.2 ml) containing various concentrations of puromycin and 3794 cpm of fMet fragment were incubated for 5 min. along with blanks lacking puromycin. The appropriate blank value was subtracted from that of each aliquot. The units of the ordinate are reciprocal of cpm in the ethyl acetate aliquot minus blank and the units of the abscissa are reciprocal molar concentration of puromycin.

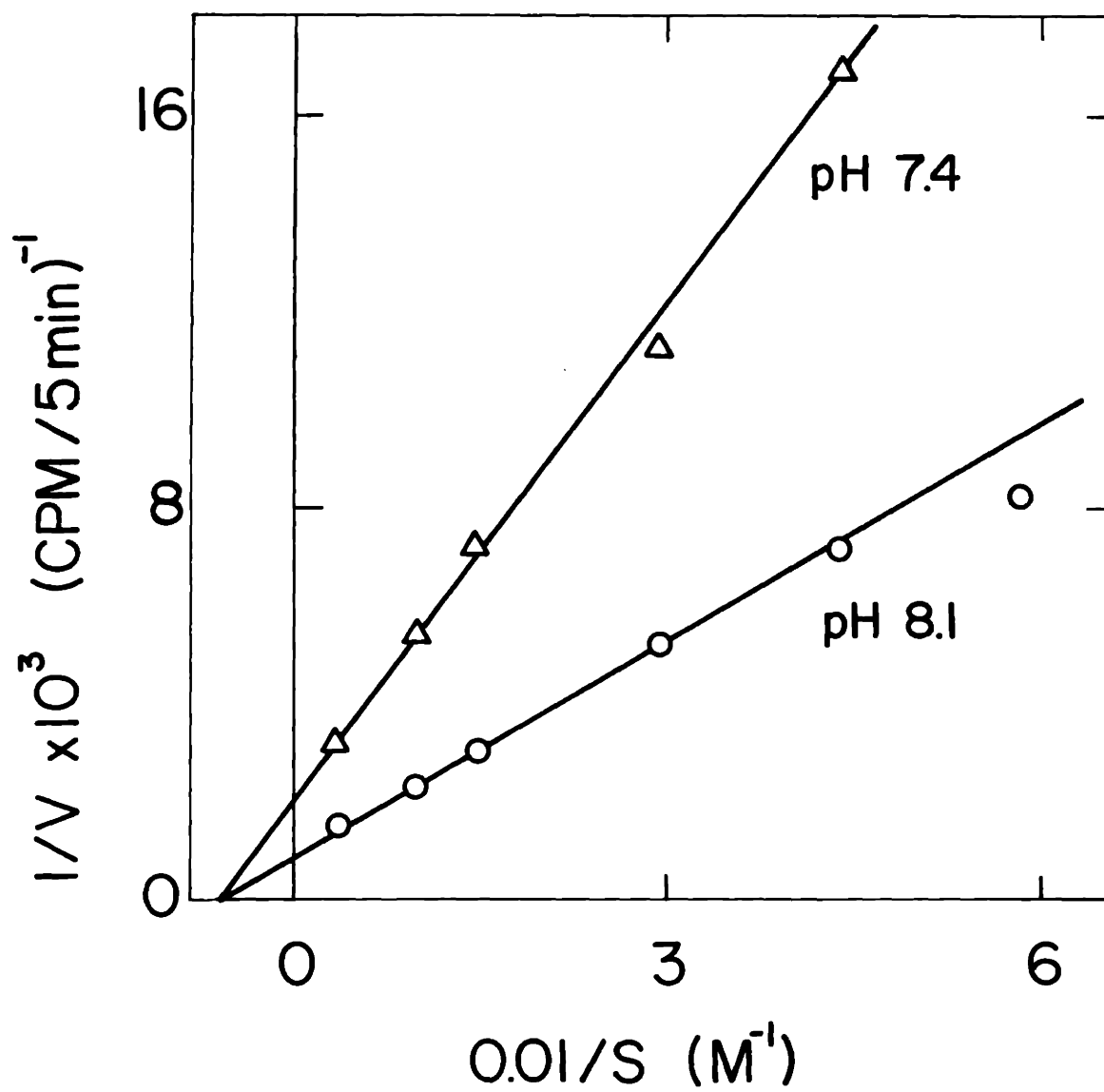


Figure 11

be interpreted in the simplest way, the ionizing group appears not to be involved in the binding of puromycin to the enzyme. The effect of pH on the V_{max} is difficult to interpret because the apparent V_{max} as determined here is a function of the binding constant of the fMet fragment (Eq. 2) as well as the V_{max} . Therefore the ionizing group could be involved either in the binding of the fMet fragment or in the rate determining catalytic step. It can be stated that the data are consistent with the involvement in the rate determining catalytic step of an imidazole residue or α -amino group, but that this is not a unique interpretation.

Regardless of the above argument, the similarity of the pH dependence of the ψ hydroxypuromycin reaction to the puromycin reaction is further evidence that both reactions are catalyzed by the same enzyme, namely peptidyl transferase.

Reaction of fMet-tRNA

The system in which the above results were obtained involves the simplest peptidyl transferase catalyzed reaction, in terms of substrate structures and cofactor requirements. In addition to reacting with the fMet fragment, ψ hydroxypuromycin is also able to react with fMet-tRNA in a more natural system in which fMet-tRNA is bound to ribosomes in the presence of a crude initia-

tion factor preparation, GTP, and as message the RNA of bacteriophage R17. A similar reaction of puromycin has been described by Bretscher and Marcker (1966) and by Zamir, et al, (1966). This reaction takes place in the absence of methanol. It is again assayed by ethyl acetate extraction. As shown in Table 2 the reaction requires ribosomes and is not catalyzed by postribosomal supernatant. Peptidyl transferase is again implicated by the inhibition of the reaction by chloramphenicol and gougerotin. The blank values in Table 2 are much lower than those observed in the fMet fragment reaction because the fMet-methylester formed in the fragment system in the presence of methanol is not formed in this system.

Electrophoretic analysis of the ethyl acetate extractable product of the reaction between fMet-tRNA and ψ hydroxypuromycin is shown in Figure 12. Like the fragment reaction product, this material has a mobility which is very similar to that of fMet-puromycin (formed in the same system) throughout the pH range 3.0 to 4.5. Only the extremes of this range are shown in Figure 12. The electrophoretic properties of this product are identical to those of the fragment reaction product.

Furthermore, as shown in panel b of Figure 12, the product formed in the presence of ψ hydroxypuromycin is destroyed by alkaline treatment. Incubation with 1 M

TABLE 2
REACTION BETWEEN fMet-tRNA AND ψ HYDROXYPUROMYCIN

	<u>DPM IN ETHYL ACETATE</u>
Complete System	796
Minus ribosomes	4
Minus ribosomes plus post- ribosomal supernatant (60 μ g protein)	-7
Plus 1 mM chloramphenicol	9
Plus 1 mM gougerotin	-4

The complete system is described in Methods (fMet-tRNA reaction). The concentration of ψ hydroxy-puromycin was 1.0×10^{-3} M. Reaction was assayed by ethyl acetate extraction. The values given are dpm in the ethyl acetate aliquot after subtraction of the values obtained from appropriate blank, incubated without ψ hydroxypuromycin. The blank values ranged from 40 to 73 dpm.

FIGURE 12: ELECTROPHORETIC CHARACTERIZATION OF PRODUCTS FORMED FROM fMet-tRNA.

Products were formed from ^{35}S fMet-tRNA in the R17 RNA directed system described in Methods. The concentration of fMet-tRNA was 9.3 A_{260} units/ml and the concentrations of puromycin (Sample P) and ψ hydroxypuromycin (Sample O) were both 5×10^{-4} M. The ethyl acetate layer was dried under reduced pressure and the material redissolved in ethanol. Aliquots of these ethanol solutions were incubated in 1 M aqueous triethylamine (pH about 12.5) for 30 min. at 37° C. These triethylamine-treated samples are designated in (b) by P' and O', respectively. Aliquots of the products were subjected to electrophoresis for 205 min. (a) or 90 min. (b) at 17 volts/cm in sodium formate buffers, pH 3.0 (a) or 4.2 (b) both at an ionic strength of 0.2, using Brinkmann cellulose thin layer plates. Radioactivity was located by autoradiography on Kodak Royal Blue X-ray film. Standard N-formylmethionine was located with the platonic iodide reagent of Toennies and Kolb (1951). In panel (a) the cathode is at the top and in panel (b) the anode is at the top. The sample labelled U is (^{14}C)uridine used to mark the origin.

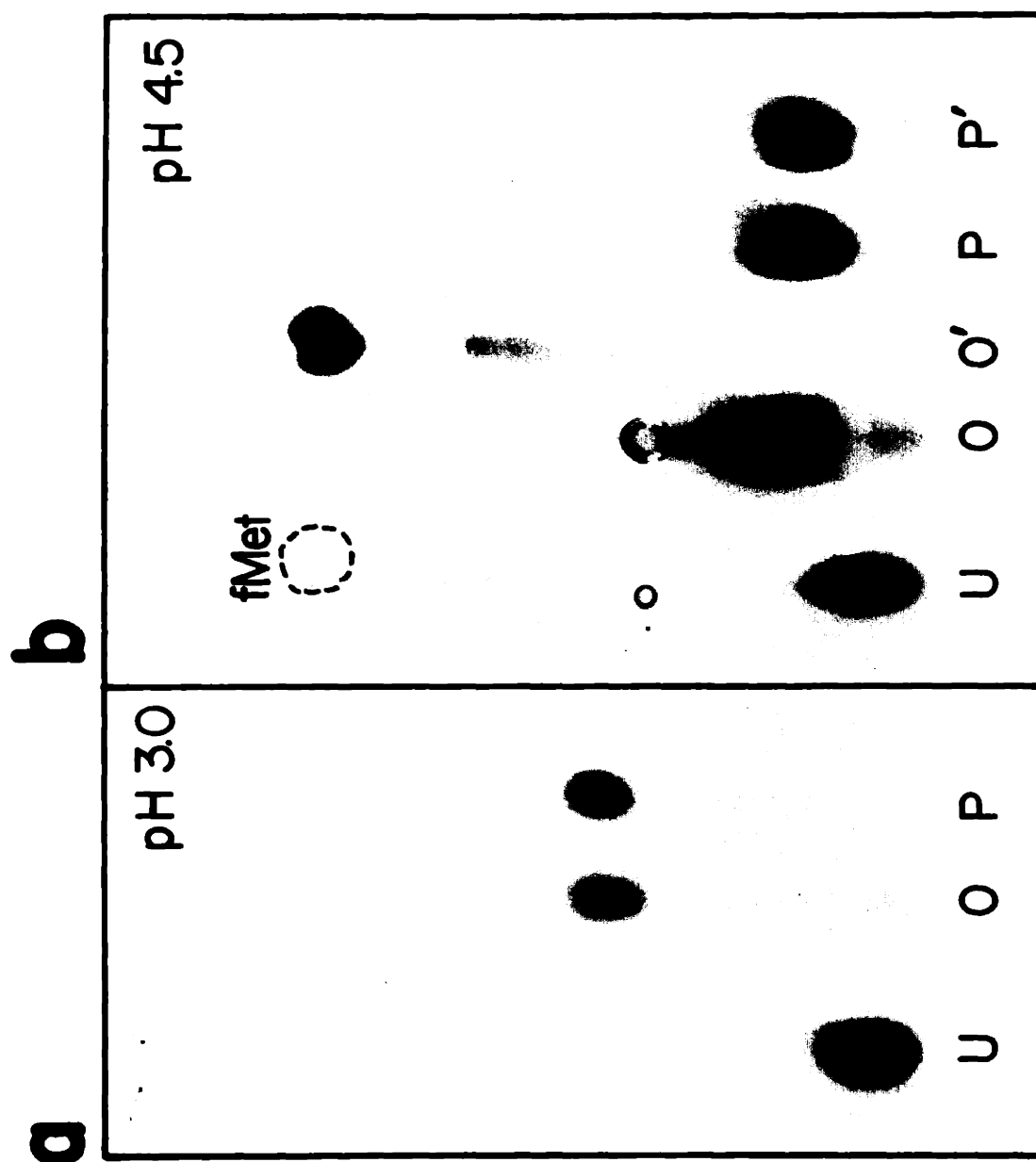


Figure 12

triethylamine (pH 12.5) for 30 min. at 37° C hydrolyzes it completely and liberates formylmethionine. fMet-puromycin is not affected by this treatment. The product is also labile under milder alkaline conditions, as shown in Figure 13. It is hydrolyzed rapidly and nearly completely at pH 9.0. Similar lability was observed for the fragment system product (Figure 7). From the data of Figure 13 it is seen that fMet- ψ hydroxypuromycin has a half life of 12 min. at pH 9.0, 30° C, in 0.04 M Tris-HCl. For comparison, the fMet-methylester formed in the fragment system has a half life of 130 min. and formylmethionyl-adenosine, formed by pancreatic RNase digestion of fMet-tRNA, has a half life of 3 min. under these conditions. Thus it can be concluded that fMet and ψ hydroxypuromycin are joined by an ester bond which is more stable than the fMet-adenosine bond in fMet-tRNA, but is considerably more labile than a simple alkyl ester like the methylester of fMet.

Thus fMet- ψ hydroxypuromycin can be formed in an aqueous system with whole fMet-tRNA. It could be argued that the reaction of ψ hydroxypuromycin in the fragment system does not reflect a physiologically relevant property of peptidyl transferase because it takes place in the presence of 50% methanol which may distort the enzyme in some way so as to alter its specificity. This argument

FIGURE 13: ALKALINE HYDROLYSIS OF fMet-
 ψ HYDROXYPUROMYCIN.

Products were formed from (^3S)fMet-tRNA in the R17 RNA-directed system, as described under Methods. The concentration of fMet-tRNA was $9.3 A_{260}$ units/ml and the concentration of puromycin or ψ hydroxypuromycin was 5×10^{-4} M. Products were extracted with ethyl acetate, the ethyl acetate layer dried under reduced pressure, and the material redissolved in ethanol. Aliquots of these solutions were mixed with 0.04 M Tris-HCl buffer at the pH indicated and incubated at 30°C . (Final ethanol concentration 1%.) Aliquots were removed at various times for assay by ethyl acetate extraction as described under Methods. Dotted Line: fMet-puromycin. Solid Lines: fMet- ψ hydroxypuromycin.

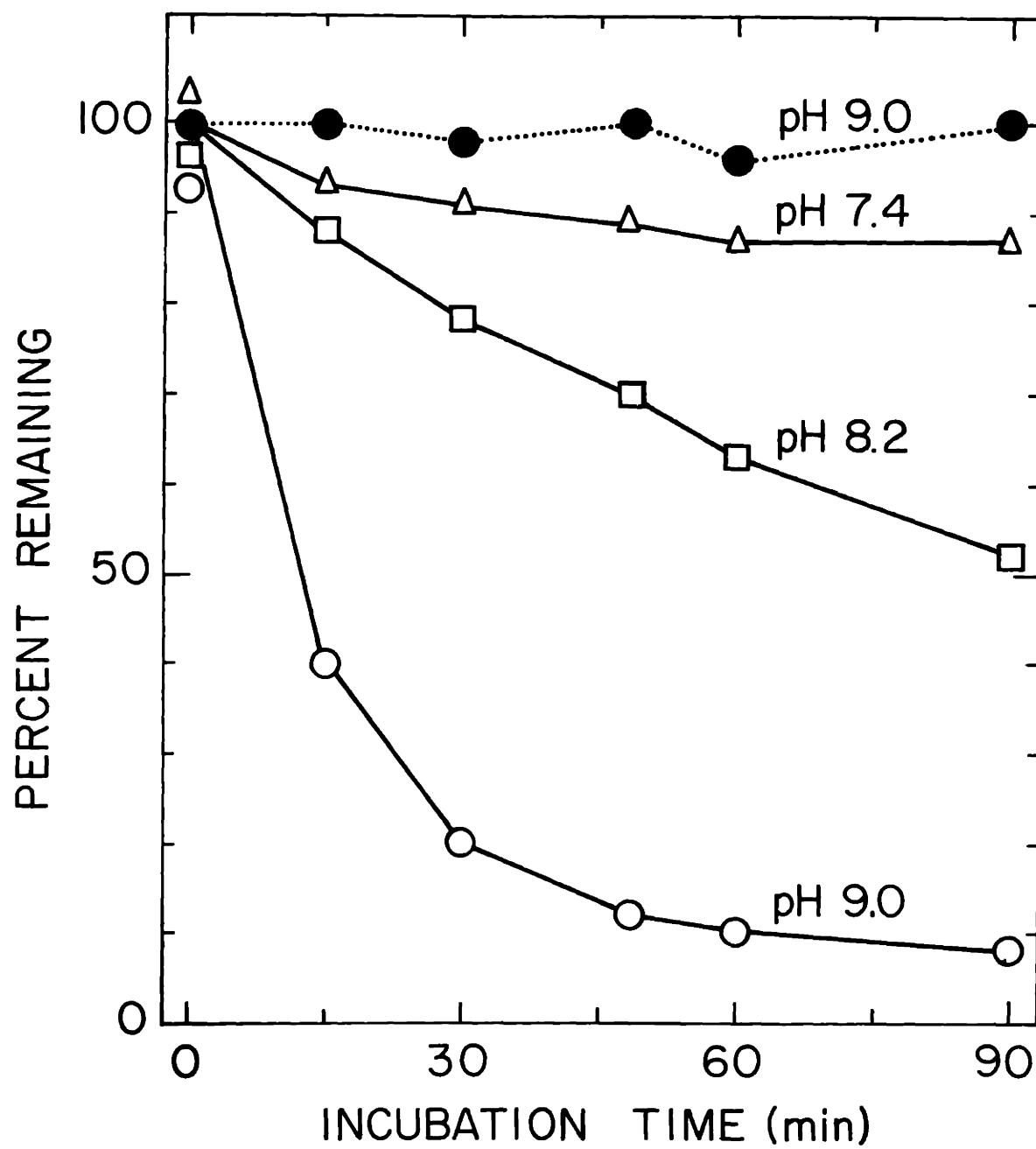


Figure 13

is not valid because the formation of fMet- ψ hydroxypuromycin takes place in aqueous solution as well as in 50% methanol.

Position of the reactive hydroxyl

The above evidence indicates that the product of reaction ^{between} fMet-hexanucleotide fragment or fMet-tRNA and ψ hydroxypuromycin contains both formylmethionine and ψ hydroxypuromycin, joined by an ester bond. Since the formation of fMet- ψ hydroxypuromycin is catalyzed by ribosomal peptidyl transferase, the same enzyme which catalyzes the formation of fMet-puromycin, it is likely that, by analogy to puromycin, the reactive group is located at the α position of the acyl moiety of ψ hydroxypuromycin. However, though the above data indicate that a hydroxyl group of ψ hydroxypuromycin is involved, it is not possible to determine on that basis which of the three hydroxyl groups (at position 2', 5', or α) it is. fMet esters at any of these three positions would be expected to have similar electrophoretic mobilities. The rate of mild alkaline hydrolysis cannot be used to identify the product in the absence of standards for comparison, since it is difficult to predict the relative labilities of the three kinds of esters.

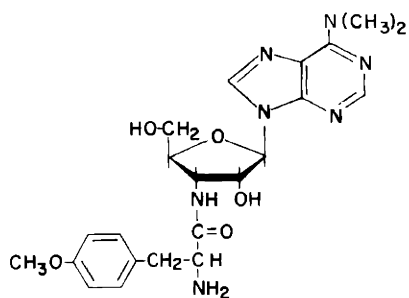
In order to determine whether the 2'- and 5'-hydroxyl groups are reactive in this system a number of puromycin

analogs were examined in which the α -amino group is replaced by an inert group, but in which the 2'- and 5'-hydroxyls are unaltered. If it is the 2'- or 5'-group on ψ hydroxypuromycin which is reactive then replacement of the α -hydroxyl group by an inert group should not prevent a similar reaction from occurring, provided the change at the α -position does not prevent binding of the puromycin analog to the enzymatic active site. However, no such reaction was observed with any of these analogs. Their inactivity was not due solely to lack of affinity for the enzyme since some of them were shown to be able to bind to the enzyme, acting as competitive inhibitors of the puromycin reaction. This indicates that the reactive group is the α -hydroxyl rather than the 2'- or 5'-group.

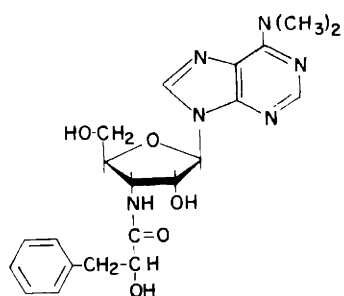
The structures of the inert analogs are shown in Figure 14. The α -amino group of puromycin is replaced by a hydrogen atom in deaminopuromycin, by a chlorine atom in α -chloropuromycin, and by a methoxy group in α -methoxypuromycin. The α -amino group is acetylated in N-acetylpuromycin. Deaminopuromycin, α -chloropuromycin, and α -methoxypuromycin were all synthesized by joining puromycin aminonucleoside to the appropriate acid. The acids used in the syntheses of α -chloropuromycin and α -methoxypuromycin were racemic (D, L) mixtures. These

FIGURE 14: STRUCTURES OF THE ANALOGS OF
PUROMYCIN.

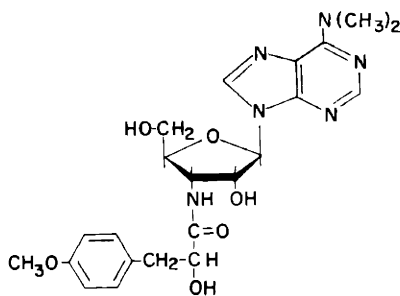
α -Hydroxypuromycin was formed by nitrous acid treatment of puromycin as described in the text. N-acetylpuromycin was synthesized from puromycin as described by Neumann, et al, (1969). ψ Hydroxypuromycin was synthesized from puromycin aminonucleoside and L-phenyllactic acid. The other analogs were synthesized from puromycin aminonucleoside and the appropriate (racemic [D, L] if asymmetric) acid.



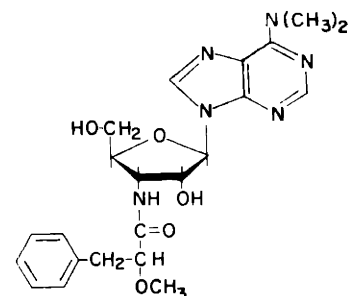
Puromycin



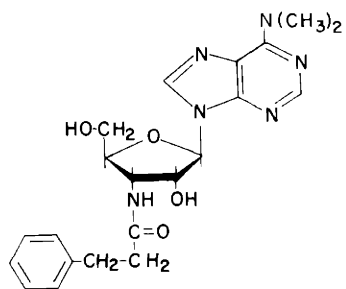
ψ-Hydroxypuromycin



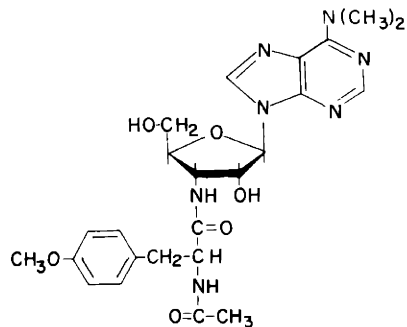
α-Hydroxypuromycin



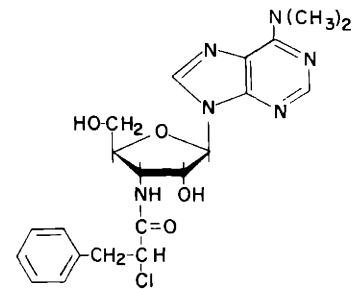
α-Methoxypuromycin



Deaminopuromycin



N-Acetylpuromycin



α-Chloropuromycin

Figure 14

three analogs, like ψ hydroxypuromycin, all lack the p-methoxy group of the acyl portion. N-acetyl puromycin was made by acetylating puromycin, and does have the p-methoxy group.

Figure 15 shows that in the fMet-hexanucleotide fragment system there is no stimulation of the appearance of ethyl acetate extractable material in the presence of 5.4×10^{-3} M deaminopuromycin. Electrophoretic analysis of the ethyl acetate extractable material formed in the presence of deaminopuromycin, shown in Figure 16 (sample D), reveals that only the fMet-methylester, which is also formed in the blank (without puromycin or deaminopuromycin), appears. There is no radioactivity which moves with fMet-puromycin, in contrast to the results with ψ hydroxypuromycin (Figure 6). Thus deaminopuromycin appears to be totally unreactive in this system.

It could be argued that the inactivity of deaminopuromycin is due to an inability to bind to the enzyme rather than deletion of the reactive group. In order to determine whether deaminopuromycin has any affinity for the enzymatic active site, its effect on the puromycin reaction was examined. If deaminopuromycin is unable to bind to the enzyme it should not affect the puromycin reaction, but if it does have affinity for the enzyme its binding might block the access of puromycin and it

FIGURE 15: TIME COURSE OF THE fMet FRAGMENT
REACTION WITH DEAMINO PUROMYCIN.

Reaction conditions and assay were standard in the fragment system. The final concentration of deamino puromycin was 5.4×10^{-3} M and the concentration of puromycin was 2.7×10^{-4} M. The "blank" sample was incubated without puromycin or deamino puromycin. Each point represents a 0.1 ml aliquot which contained a total of 9130 dpm of (^{35}S) fMet oligonucleotide fragment. The ordinate value is [(dpm in 1 ml ethyl acetate aliquot) $\times 150$]/9130.

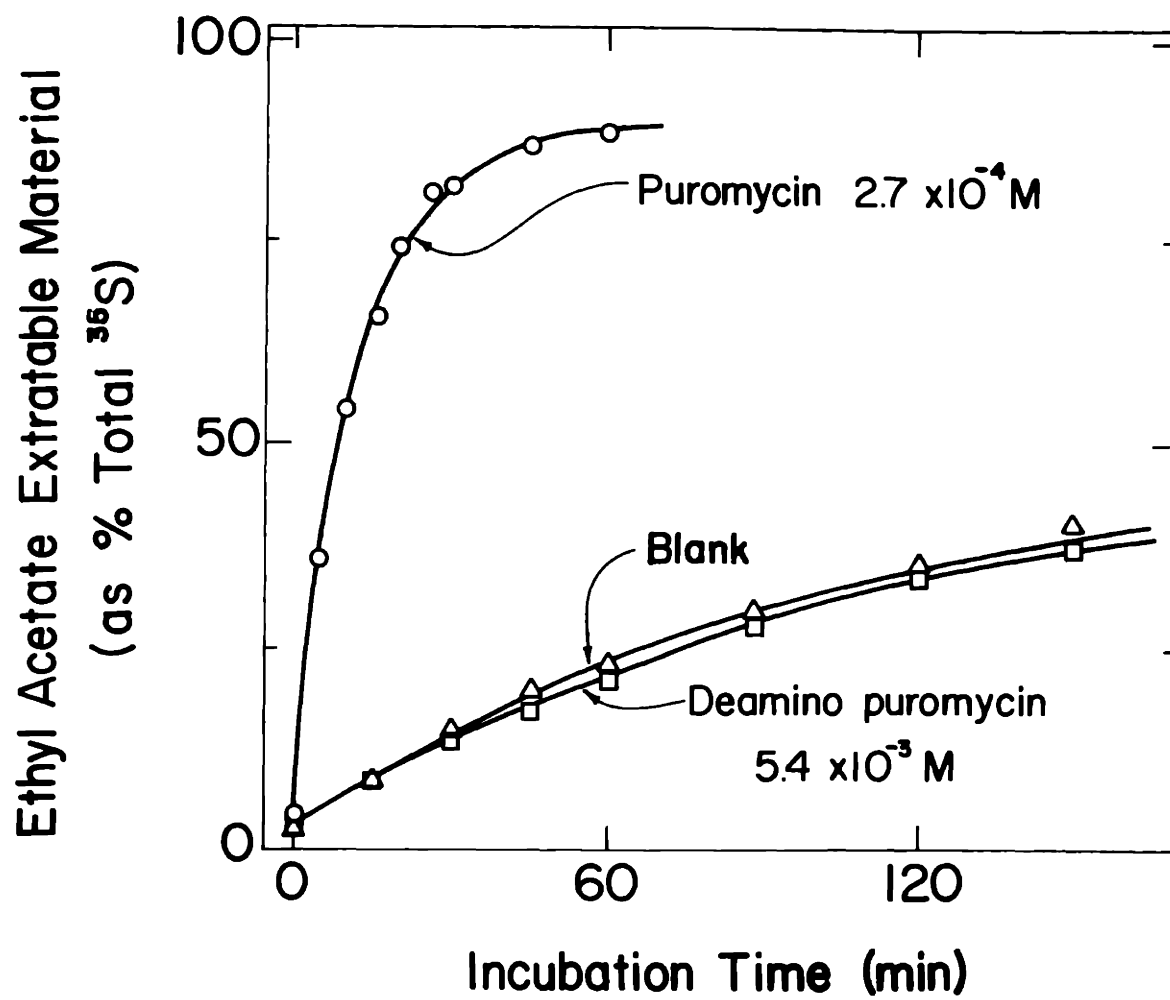


Figure 15

FIGURE 16: ELECTROPHORETIC ANALYSIS OF ETHYL ACETATE EXTRACTABLE MATERIAL FORMED IN THE FRAGMENT SYSTEM IN THE PRESENCE OF DEAMINO PUROMYCIN AND N-ACETYL PUROMYCIN.

Products were formed as described in the legend to Figure 6. The concentration of deamino puromycin (Sample D) was 2×10^{-3} M, the concentration of N acetyl puromycin (Sample A) was 6×10^{-3} M, and the concentration of puromycin (Sample P) was 2.5×10^{-4} M. Sample B was a blank incubated without puromycin or analogs. Sample U is ^{14}C uridine used to mark the origin. Electrophoresis was at pH 3.0 under conditions described in Figure 6 with the cathode at the top of the autoradiogram as shown.

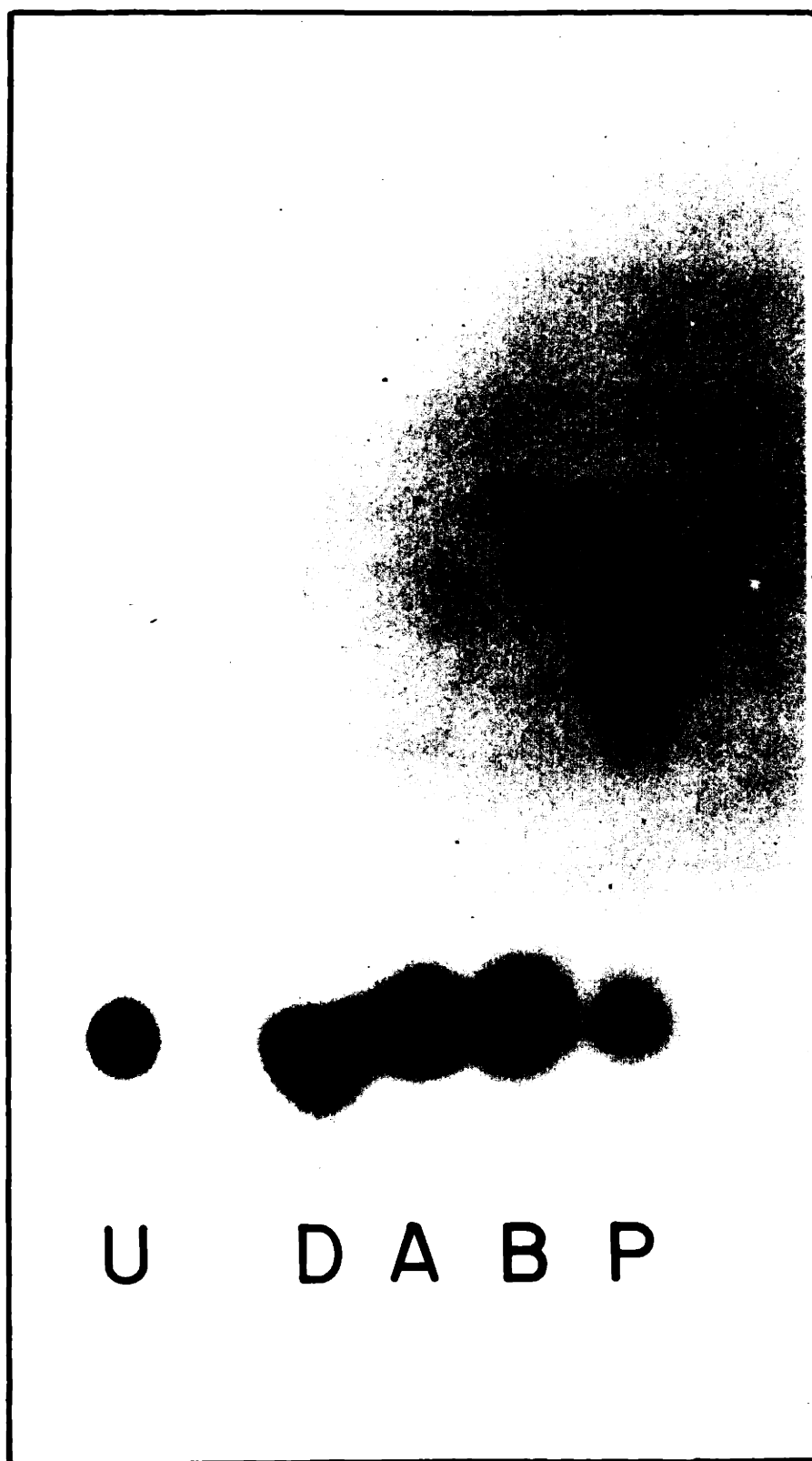


Figure 16

could act as a competitive inhibitor of the puromycin reaction.

Figure 17 shows the rate of the puromycin reaction as a function of the concentration of puromycin in the presence and absence of deaminopuromycin. As usual the data are expressed in Lineweaver-Burk plots. The effect of 4.4×10^{-3} M deaminopuromycin is to inhibit the puromycin reaction markedly. The data in the presence and absence of deaminopuromycin appear to fit straight lines which intersect on the ordinate. Thus the inhibition by deaminopuromycin appears to be competitive with puromycin.

Assuming the simplest kinetic model, as discussed above, it is possible to use these data to calculate the apparent binding constant of deaminopuromycin to the enzyme. The value obtained is $K_i = 1.9 \times 10^{-3}$ M. This is roughly twice the dissociation constant of ψ hydroxypuromycin, measured in its reaction or by its inhibition of the puromycin reaction. The concentration of deaminopuromycin used in the electrophoretic analysis of Figure 16 was 2.0×10^{-3} M. At this concentration deaminopuromycin binds extensively (roughly half saturation) to peptidyl transferase. Therefore its inactivity is not due to lack of affinity to the enzyme. It is therefore implied that the compound is inactive because the reactive group is missing, so the reactive group in ψ hydroxypuromycin must

FIGURE 17: INHIBITION OF THE PUROMYCIN
REACTION BY DEAMINO PUROMYCIN.

Reaction conditions were standard in the fragment system. Aliquots (0.2 ml) containing 8100 cpm of fMet fragment and various concentrations of puromycin only (o) or various concentrations of puromycin plus 4.4×10^{-3} M deamino puromycin, (Δ) and blanks lacking puromycin were incubated for 3 min., then assayed by ethyl acetate extraction in the standard manner. The data are presented as Lineweaver-Burk plots in which the units of the ordinate are reciprocal of cpm in the ethyl acetate aliquot minus blank and the units of the abscissa are reciprocal molar concentration of puromycin.

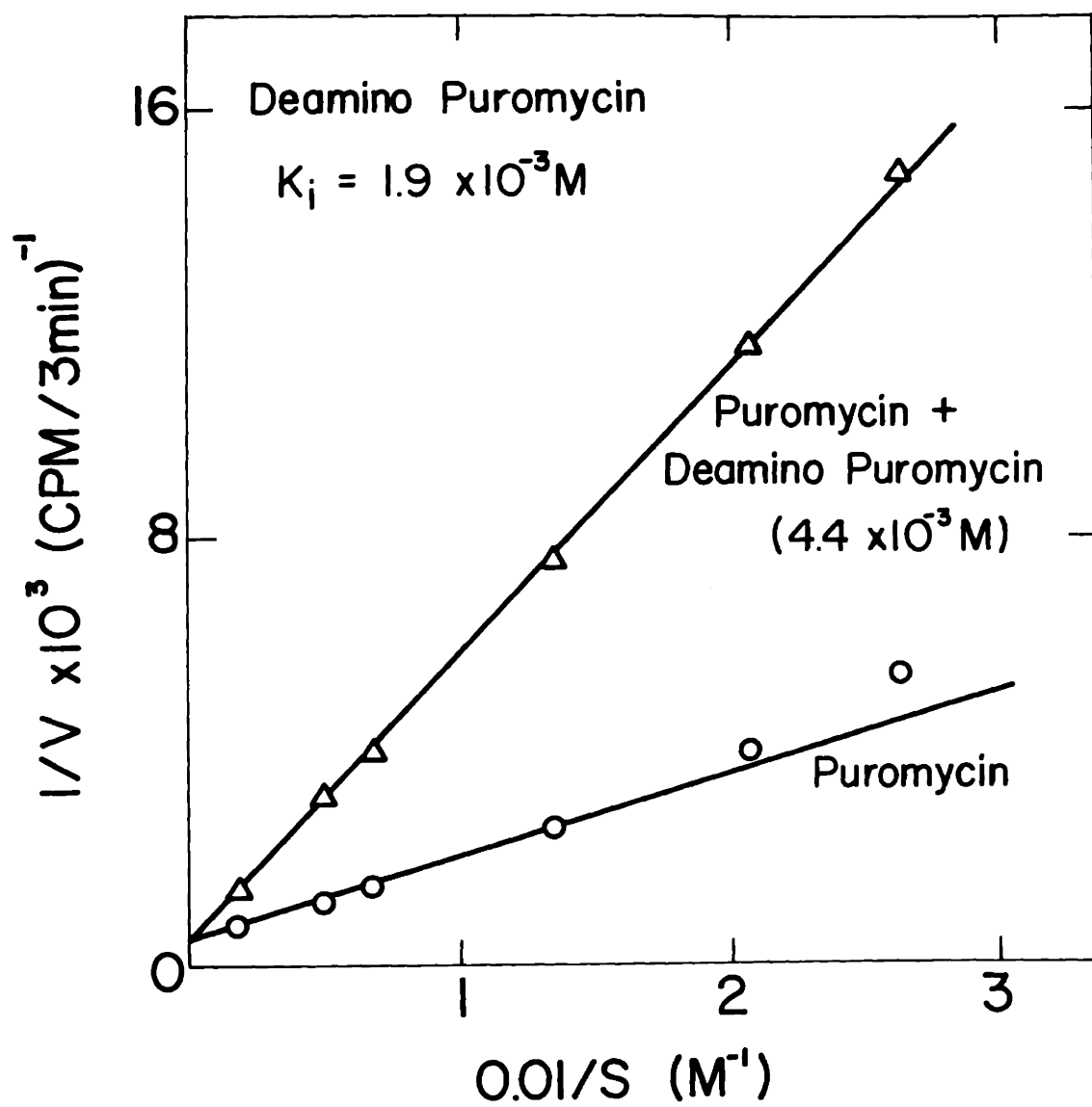


Figure 17

be the α -hydroxyl.

There is one reservation with respect to this conclusion which should be stated. The binding which is indicated by inhibition of the puromycin reaction is not necessarily binding in an orientation which would allow reaction. The symmetric α carbon in deaminopuromycin may allow binding in a slightly improper orientation which would prevent reaction even if the reactive group itself were present. The other puromycin analogs discussed below are asymmetric at the α carbon (though most are racemic mixtures) and contain various electronegative atoms attached to the α carbon. It is unlikely, though possible, that this condition affects all of the four analogs but not puromycin or ψ hydroxypuromycin.

Also shown in Figure 16 is the electrophoretic analysis of the ethyl acetate extractable material formed in the presence of 6×10^{-3} M N-acetylpuromycin. Again there is no radioactivity in region near fMet-puromycin. Only the fMet-methylester, which is also formed in the blank, is present. Therefore, N-acetyl puromycin is also inactive in the standard fMet-hexanucleotide fragment system.

At a concentration of 5×10^{-3} M, no activity of N-acetylpuromycin is observed under a variety of conditions. There is no stimulation of the formation of ethyl acetate extractable radioactivity in the fMet fragment system at

Mg²⁺ concentrations between 0 and 30 mM, at temperatures between 0° C and 23° C, in either 50% methanol or 50% ethanol. (Puromycin and ψ hydroxypuromycin are active in ethanol.)

The inactivity of N-acetylpuromycin is not due to lack of affinity for the enzyme. Figure 18 shows that N-acetylpuromycin, at 5×10^{-3} M, inhibits the puromycin reaction. The inhibition appears to be competitive with puromycin. The inhibitor dissociation constant, K_i , for N-acetylpuromycin, calculated from the data in Figure 18 is 6×10^{-3} M. Thus N-acetylpuromycin is able to bind to the active site and inhibit the puromycin reaction at a concentration where it is not itself reactive.

The fact that the inhibition by N-acetylpuromycin appears to be simply competitive with puromycin might seem unexpected. The amide linkage at the α -amino group makes N-acetylpuromycin an analog of peptidyl-tRNA in much the same manner that the N-formylmethionyl-hexanucleotide is such an analog. Therefore it might be expected to bind also to donor site on the enzyme, interfering with the binding of the fMet-hexanucleotide. This would be manifest as a change in the V_{max} for puromycin in Figure 18, so that the inhibition by N-acetylpuromycin would not appear simply competitive. This behavior was not observed. It appears that in spite of its acylated amino group, N-acetyl-

FIGURE 18: INHIBITION OF THE PUROMYCIN REACTION BY N-ACETYL PUROMYCIN.

Reaction conditions were standard in the fragment system. Aliquots (0.2 ml) containing 4652 cpm of fMet fragment and various concentrations of puromycin only (o) or various concentrations of puromycin plus 5×10^{-3} M N-acetyl puromycin (Δ), and blanks lacking puromycin were incubated for 5 min. then assayed by ethyl acetate extraction in the standard manner. The data are presented as Lineweaver-Burk plots in which the units of the ordinate are reciprocal of cpm in the ethyl acetate aliquot minus control and the units of the abscissa are reciprocal molar concentration of puromycin.

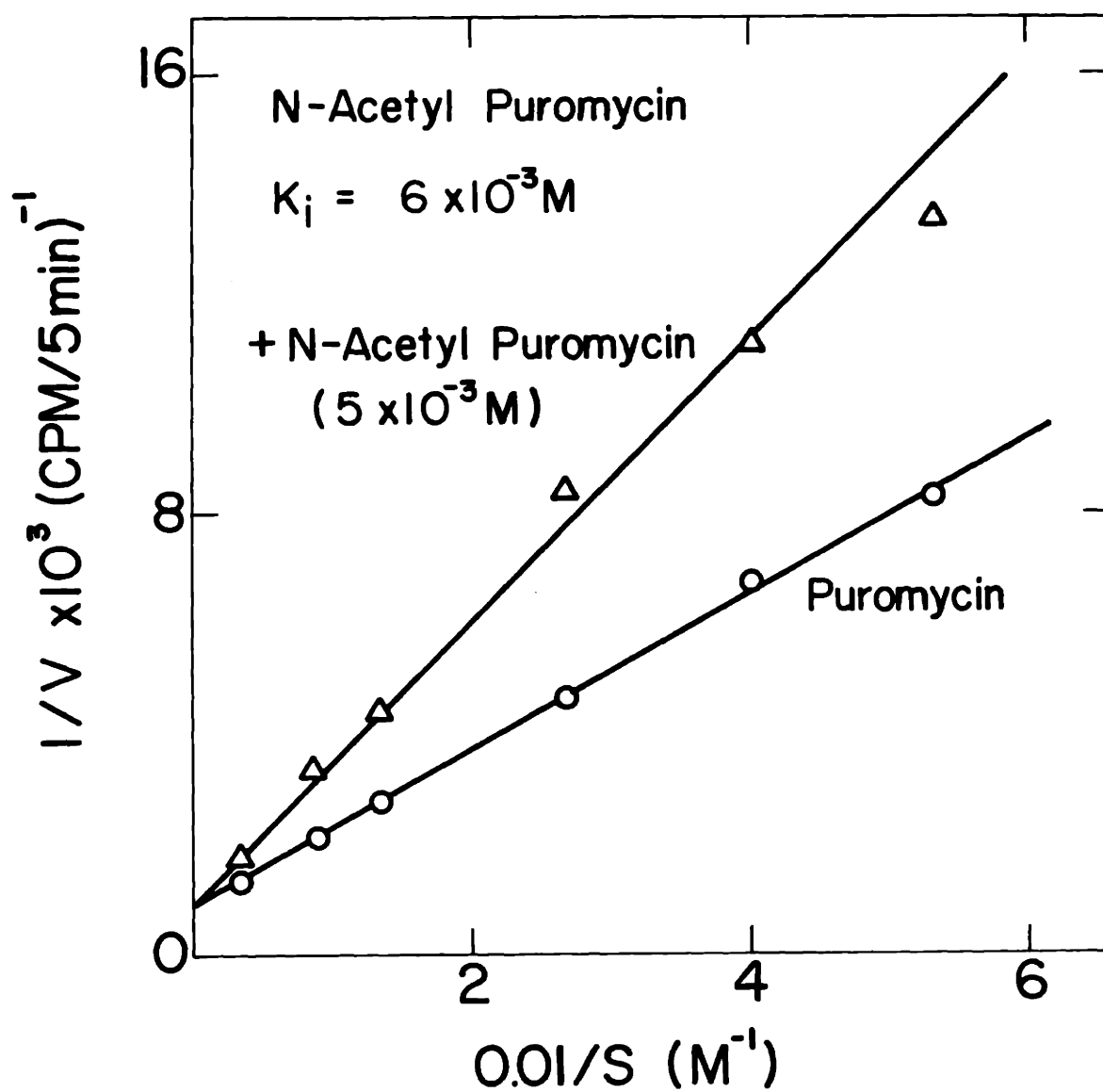


Figure 18

puromycin has a higher affinity for the peptidyl transferase acceptor site than for the donor site. This is consistent with the observation of Monro, et al, (1968) that the minimal structure required of a donor substrate is a trinucleotide. Thus while the acceptor substrate interacts with the enzyme significantly at its terminal adenosine and aminoacyl regions, important interactions of the donor substrate occur at other nucleotides as well.

Another puromycin analog which is not active in the fMet fragment system is α -chloropuromycin. At 7×10^{-3} M, α -chloropuromycin does not stimulate the formation of ethyl acetate extractable material, but as shown in Figure 19 it inhibits the puromycin reaction at a lower concentration, 3.3×10^{-3} M. Inhibition was measured at only one concentration of puromycin because the supply of α -chloropuromycin was exhausted. Therefore it is impossible to determine whether this inhibition is competitive. It is not unreasonable to assume that it is competitive in view of the behavior of the other puromycin analogs. Making this assumption (drawing a line through the inhibited point which intersects the line determined by the uninhibited puromycin data at the ordinate) it is possible to calculate $K_i = 3.5 \times 10^{-3}$ M for α -chloropuromycin.

Another analog which is not reactive in the fMet fragment system is α -methoxypuromycin. This compound gives

FIGURE 19: INHIBITION OF THE PUROMYCIN REACTION
BY α -CHLORO PUROMYCIN.

Reaction conditions were standard in the fragment system. Aliquots (0.2 ml) containing 3009 cpm of fMet fragment and various concentrations of puromycin were incubated for 5 min. One sample contained, in addition to puromycin, 3.3×10^{-3} M α -chloro puromycin. A blank was incubated without puromycin and its value was subtracted from the others. The units of the ordinate are reciprocal of cpm in the ethyl acetate layer minus blank and the units of the abscissa are reciprocal of molar concentration of puromycin.

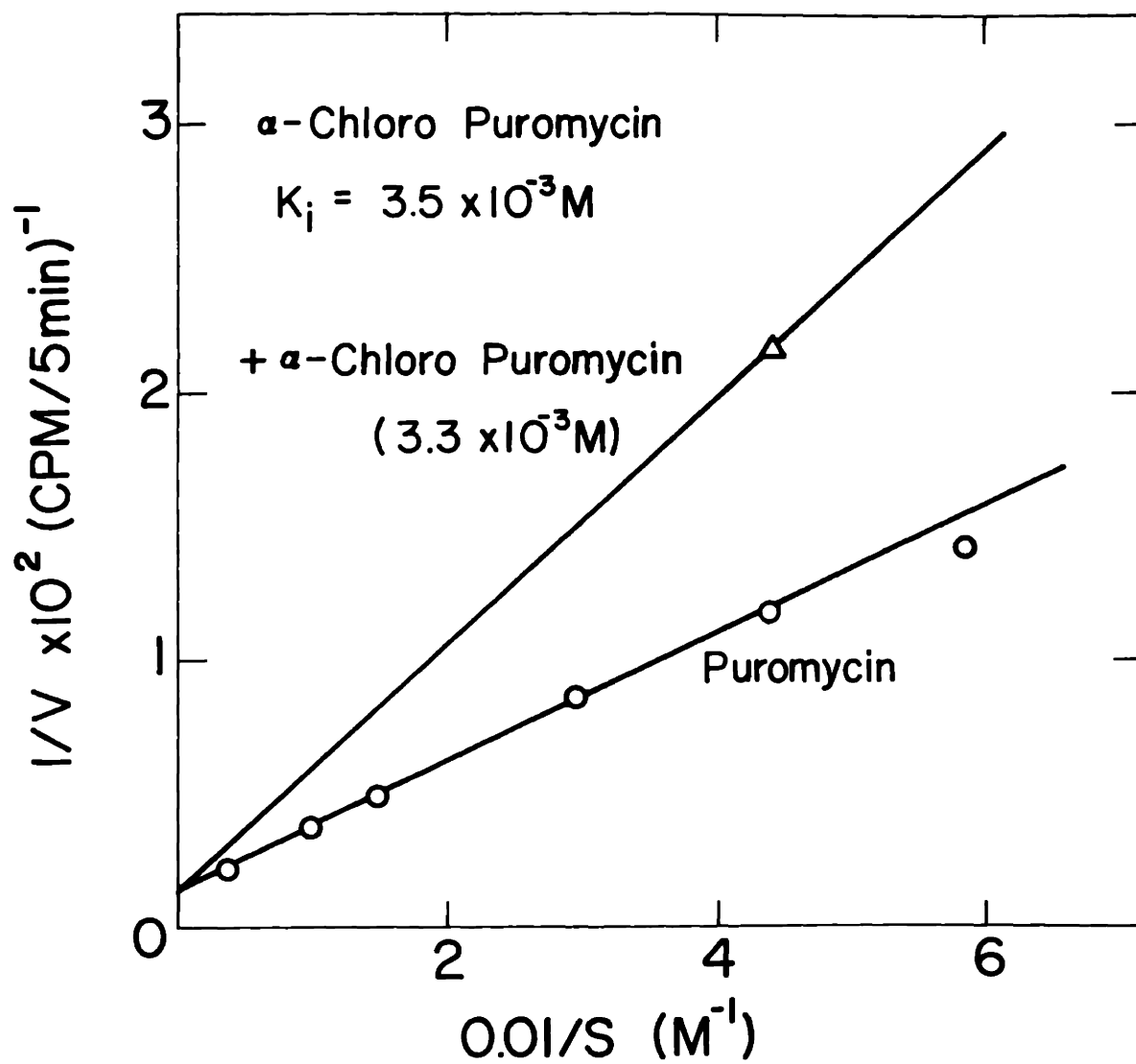


Figure 19

no stimulation of formation of ethyl acetate extractable material at concentrations up to 2×10^{-2} M. Its effect on the puromycin reaction was not determined.

Thus none of these puromycin analogs is reactive under conditions where ψ hydroxypuromycin can react. Three of them were shown to inhibit the puromycin reaction, two of them competitively, at concentrations where they cannot themselves react. Therefore their inactivity is not due to lack of affinity for the enzyme, but must be attributed to alteration of the reactive group itself. The reactive group in ψ hydroxypuromycin must be the α -hydroxyl rather than the 2'- or 5'- groups because the 2'- and 5'- groups are unaltered in these inactive compounds.

Nitrous acid deaminated puromycin

As described under Materials, treatment of puromycin with nitrous acid results in the formation of a mixture of deaminated products which can be separated by chromatography on silica gel. On the basis of the well-known properties of this reaction it can be predicted that one of these products should be identical to puromycin but with a hydroxyl group substituted for the α -amino group of puromycin. The other products would be expected to arise by rearrangement of the intermediate carbonium ion like those observed by Roberts and Regan (1953) in a similar reaction, and by racemization at the α -carbon. Of the three components

which can be separated on silica gel chromatography, only one is reactive in the fMet-hexanucleotide fragment system. It behaves identically to ψ hydroxypuromycin in this respect. The structure of this compound is not known, but because of this similar activity it is presumably identical to ψ hydroxypuromycin except for the presence of the p-methoxy group which is present in puromycin but missing in ψ hydroxypuromycin. The p-methoxy group does not appreciably affect the activity of puromycin (Nathans and Niedle, 1963). This compound will be referred to as α -hydroxypuromycin.

Like ψ hydroxypuromycin, the presence of α -hydroxypuromycin in the fragment system results in a stimulation of the formation of ethyl acetate extractable radioactivity from (^{35}S)fMet-hexanucleotide fragment. As shown in Table 3 this reaction is catalyzed by ribosomes but not by post-ribosomal supernatant. It requires the presence of potassium and magnesium ions, and is inhibited by the peptidyl transferase inhibitors chloramphenicol and gougerotin.

The dependence of the rate of reaction on the concentration of α -hydroxypuromycin is shown in Figure 20. The K_m for α -hydroxypuromycin determined from this Lineweaver-Burk plot is 1.0×10^{-3} M, the same as the K_m for ψ hydroxypuromycin. The ratio of the V_{max} for α -hydroxypuromycin to that for puromycin measured in the same experi-

TABLE 3
REACTION BETWEEN fMet-HEXANUCLEOTIDE FRAGMENT
AND α -HYDROXYPUROMYCIN

	<u>DPM IN ETHYL ACETATE</u>
Complete system	250
Minus Mg^{2+}	2
Minus K^+	-6
Minus ribosomes	14
Minus ribosomes plus post- ribosomal supernatant (15 μ g protein)	-20
Plus 1 mM chloramphenicol	10
Plus 1 mM gougerotin	-4

Experimental protocol was as described in Table 1. The concentration of α -hydroxypuromycin was 6.5×10^{-4} M. The values listed are dpm in the ethyl acetate aliquot after subtraction of the appropriate blank value. The blank values ranged from 180 dpm to 220 dpm, except the sample with gougerotin, which was 100 dpm.

FIGURE 20: DEPENDENCE OF INITIAL REACTION RATE
UPON CONCENTRATION OF α -HYDROXY-
PUROMYCIN.

Reaction conditions and treatment of data were as described in Figure 3. Samples were incubated for either 7.5 min. (three highest concentrations) or 15 min. The values obtained from the 7.5 min. incubations were doubled so that all data could be expressed in the same units. Each aliquot contained 4080 cpm of formylmethionyl- T_1 fragment.

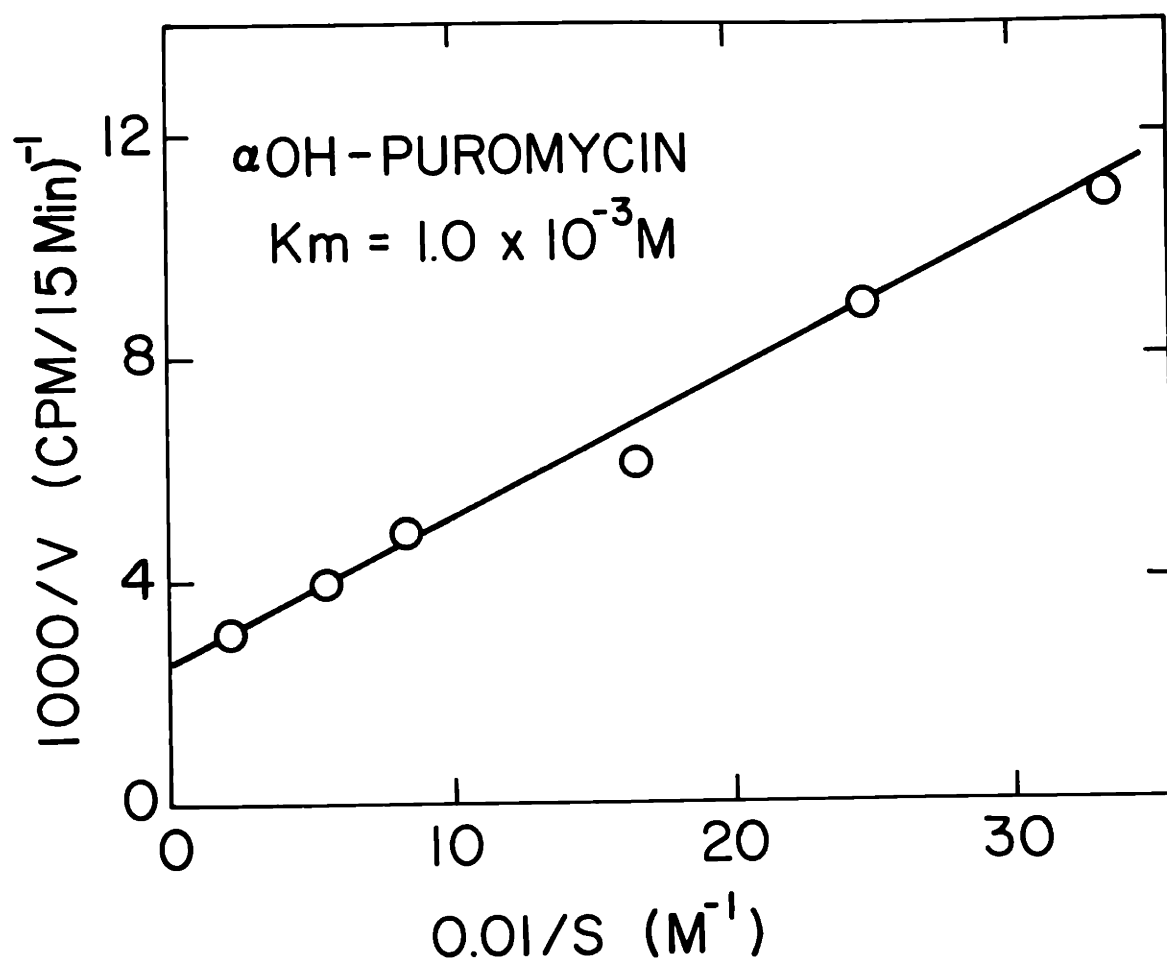


Figure 20

ment is 0.14. The analogous ratio for ψ hydroxypuromycin is 0.11.

Electrophoretic analysis (Figure 21) of the ethyl acetate extractable material formed from (^3S)fMet-hexanucleotide fragment reveals, in addition to the fMet-methylester present in the blank (B), the formation of a component which moves in the same region with fMet-puromycin (P) at pH 2.9, and like fMet-puromycin has little mobility at pH 4.3. In Figure 21 the origin (location of neutral compounds) was not located with (^{14}C)uridine as it was in Figure 6, but it can be assumed by analogy with Figure 6 that the position of the radioactivity in the blank is approximately at the origin. (This is not the point at which the material was spotted because of the endosmotic solvent flow.)

The ethyl acetate extractable product of reaction between fMet-fragment and α -hydroxypuromycin displays the same alkaline lability as fMet- ψ hydroxypuromycin. It is completely destroyed by treatment with 1 M KOH for 10 min. at 23° C. Furthermore it is hydrolyzed at pH between 8 and 9 at approximately the same rate as fMet- ψ hydroxypuromycin.

Thus nitrous acid treatment of puromycin creates a compound which behaves like ψ hydroxypuromycin in the fragment system. It participates in a ribosome-catalyzed reaction with fMet-hexanucleotide fragment to form a

FIGURE 21: ELECTROPHORETIC ANALYSIS OF ETHYL
ACETATE EXTRACTABLE MATERIAL FORMED
IN THE FRAGMENT SYSTEM IN THE PRESENCE
OF α -HYDROXYPUROMYCIN.

Products were formed in the standard fragment system with 55,000 dpm/ml (^{35}S) fMet-oligonucleotide fragment. The sample labelled P contained 3×10^{-3} M puromycin, the sample labelled O contained 6.5×10^{-4} M α -hydroxypuromycin, and the sample B (blank) contained no puromycin or α -hydroxypuromycin. After incubation for 1 hour, products were extracted with ethyl acetate in the usual manner, the ethyl acetate layer was dried under reduced pressure, and the material redissolved in ethanol. Aliquots of the products were subjected to electrophoresis in a sodium formate buffer pH 2.9 (a) or 4.3 (b) both at ionic strength 0.2 at 17 v/cm for 2 hours on Brinkmann cellulose thin layer plates. In the autoradiograms shown the cathode is at the top.

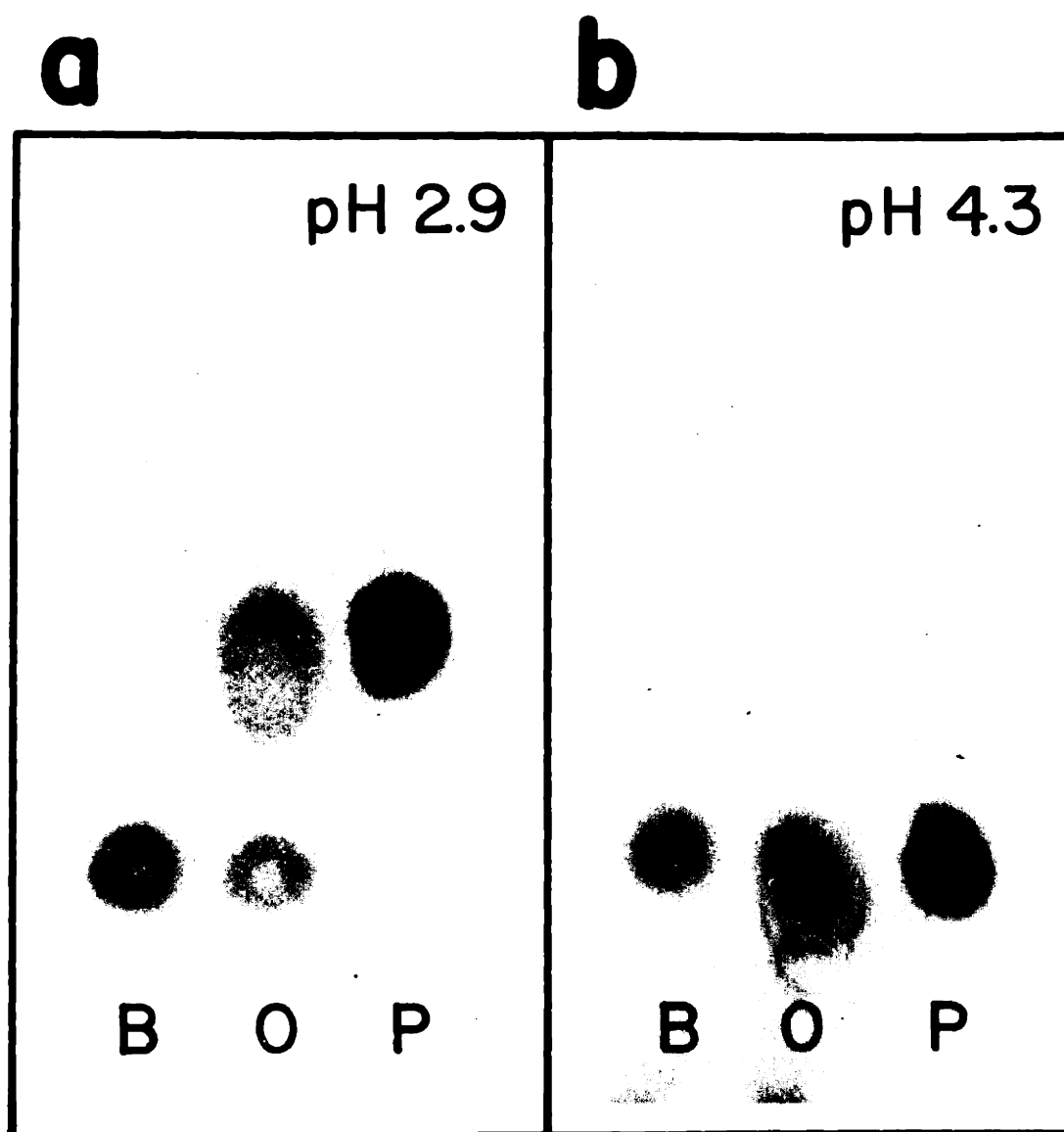


Figure 21

product which resembles fMet-puromycin but which is labile under mild alkaline conditions.

DISCUSSION

The α -hydroxyl analog of puromycin is responsible for the formation of a compound which is electrophoretically indistinguishable from formylmethionyl-puromycin. This product differs from fMet-puromycin by its lability under mildly alkaline conditions. It contains formylmethionine, which is released on alkaline hydrolysis. Its similarity to fMet-puromycin on electrophoresis and the dependence of its mobility on pH in the range near the pK_a of the puromycin purine dimethylamino group indicate that it also contains the puromycin analog. The alkaline lability of the compound is indicative of an ester linkage which must be formed between the carboxyl group of formylmethionine and one of the hydroxyl groups of ψ hydroxypuromycin.

This product can be formed from formylmethionyl-tRNA or from the fMet containing hexanucleotide fragment derived from fMet-tRNA by digestion with ribonuclease T1. The fragment reaction was studied most extensively.

The reaction is catalyzed by the same enzyme, ribosomal peptidyl transferase, which catalyzes the formation of fMet-puromycin. This is indicated by the following:

(1) The reaction is catalyzed by salt-washed ribosomes, but not by the postribosomal supernatant. (2) In the fragment system the reaction has the same requirement for magnesium and potassium ions and the same pH dependence

as the puromycin reaction. (3) It is inhibited by two specific inhibitors of peptidyl transferase, chloramphenicol and gougerotin. (4) The catalytic activities of the ribosome preparation for the ψ hydroxypuromycin and puromycin reactions undergo parallel inactivation at high temperatures. (5) The reaction is specific for the L isomer of ψ hydroxypuromycin. (6) ψ hydroxypuromycin acts as a competitive inhibitor of the puromycin reaction with a dissociation constant (K_i) equal to its K_m determined by its own reactivity.

By analogy to the puromycin reaction it is suggested that the reactive group in ψ hydroxypuromycin is the α -hydroxyl rather than the 2'- or 5'- groups. Evidence that this is the case is provided by the observation that a number of other analogs in which inert groups are substituted for the α -amino group of puromycin are inactive in spite of the fact that the 2'- and 5'- hydroxyls are unaltered in these compounds. Furthermore the inactivity of deaminopuromycin and N-acetyl puromycin (and probably α -chloropuromycin) is not due to lack of affinity for the enzyme since they are able to bind to the active site, acting as competitive inhibitors of the puromycin reaction.

It is concluded that the product fMet- ψ hydroxypuromycin has the structure shown in Figure 1. The ester linkage in this compound is formed by peptidyl transferase catalyzed

transfer of formylmethionine to the α -hydroxyl group of ψ hydroxypuromycin, presumably by a mechanism analogous to that used in normal peptide bond formation.

A compound which behaves similarly to ψ hydroxypuromycin is formed by treating puromycin with nitrous acid. Its structure is presumably the same as that of ψ hydroxypuromycin except for the presence of a methoxy group.

The kinetic constants determined for the various compounds which were examined are tabulated in Table 4.

TABLE 4

KINETIC PARAMETERS OF THE PUROMYCIN ANALOGS

<u>COMPOUND</u>	<u>α-SUBSTITUENT</u>	<u>K_m</u>	<u>K_i</u>	<u>RELATIVE V_{max}</u>
Puromycin	-NH ₂	1.9×10^{-4} M	--	1.0
ψ hydroxypuromycin	-OH	1.0×10^{-3} M	0.9×10^{-3} M	0.11
α -hydroxypuromycin	-OH	1.0×10^{-3} M	--	0.14
Deaminopuromycin	-H	--	1.9×10^{-3} M	0
N-acetylpuromycin	-NHCOCH ₃	--	6.0×10^{-3} M	0
α -chloropuromycin	-Cl	--	3.5×10^{-3} M	0

123

All constants were evaluated in the fMet fragment system as described in the text, at 0° C in 50% methanol. K_i refers to inhibition of the puromycin reaction. V_{max} is expressed as a ratio to the V_{max} for puromycin determined in the same experiment under identical conditions.

REACTIONS OF α -HYDROXYACYL-tRNAs

The results described in the previous section demonstrate that the *E. coli* ribosome can catalyze a transesterification reaction. The properties of that reaction suggest that it is catalyzed by the ribosomal enzyme which functions in peptide bond formation, namely, peptidyl transferase. Although those experiments demonstrated the ability of peptidyl transferase to form ester bonds using an α -hydroxyacyl analog of puromycin, the results left open the question of whether the ribosome is able to form ester bonds in a system in which messenger RNA is directing the synthesis of polypeptide chains under physiological conditions.

In this section the ability of peptidyl transferase to catalyze the formation of ester linkages is further demonstrated in systems analogous to polypeptide synthesis. α -Hydroxyacyl-tRNAs are utilized as acceptor substrates. This results in the incorporation of α -hydroxy acids into internal positions in a polymer, forming a polyester or a polypeptide containing ester linkages.

The α -hydroxyacyl-tRNAs are formed by nitrous acid deamination of aminoacyl-tRNAs. This conversion was first described by Hervé and Chapeville (1965), who synthesized phenyllactyl-tRNA by deaminating phenylalanyl-tRNA. A similar conversion is used in these experiments, as well

as an analogous reaction which converts alanyl-tRNA into lactyl-tRNA (Figure 22). However the deamination reactions are carried out under conditions which are somewhat milder than those used by Hervé and Chapeville, since their procedure was found to result in considerable damage to the RNA. The procedure used in these experiments (1 M NaNO_2 , 0.01 M magnesium acetate, pH 4.3 for 30 min. at 23° C) was shown by Carbon (1965) to result in the deamination, on the average, of less than 0.2 nucleotides per tRNA molecule.

Two kinds of *in vitro* systems were used in these experiments. One is similar to the well known polyuridylic acid directed system (Nirenberg and Matthaei, 1961). Phenyllactyl-tRNA, which is formed by treating Phenylalanyl-tRNA with nitrous acid, is incorporated in a ribosomal, polyuridylic acid dependent reaction into an acid precipitable polymer which appears to be a polyester. In the other system synthesis is directed by a natural messenger RNA, the RNA of bacteriophage R17. In this case α -hydroxy acids are incorporated into specific internal positions in a peptide of the phage coat protein in place of the analogous amino acids. The result is a polypeptide containing ester bonds at specific positions.

It is likely that these ester-forming reactions exactly parallel the process of polypeptide synthesis which occurs under similar conditions, and that the formation of ester bonds is catalyzed by peptidyl transferase acting similarly

FIGURE 22: SYNTHESIS OF PHENYLLACTYL-tRNA
AND LACTYL-tRNA.

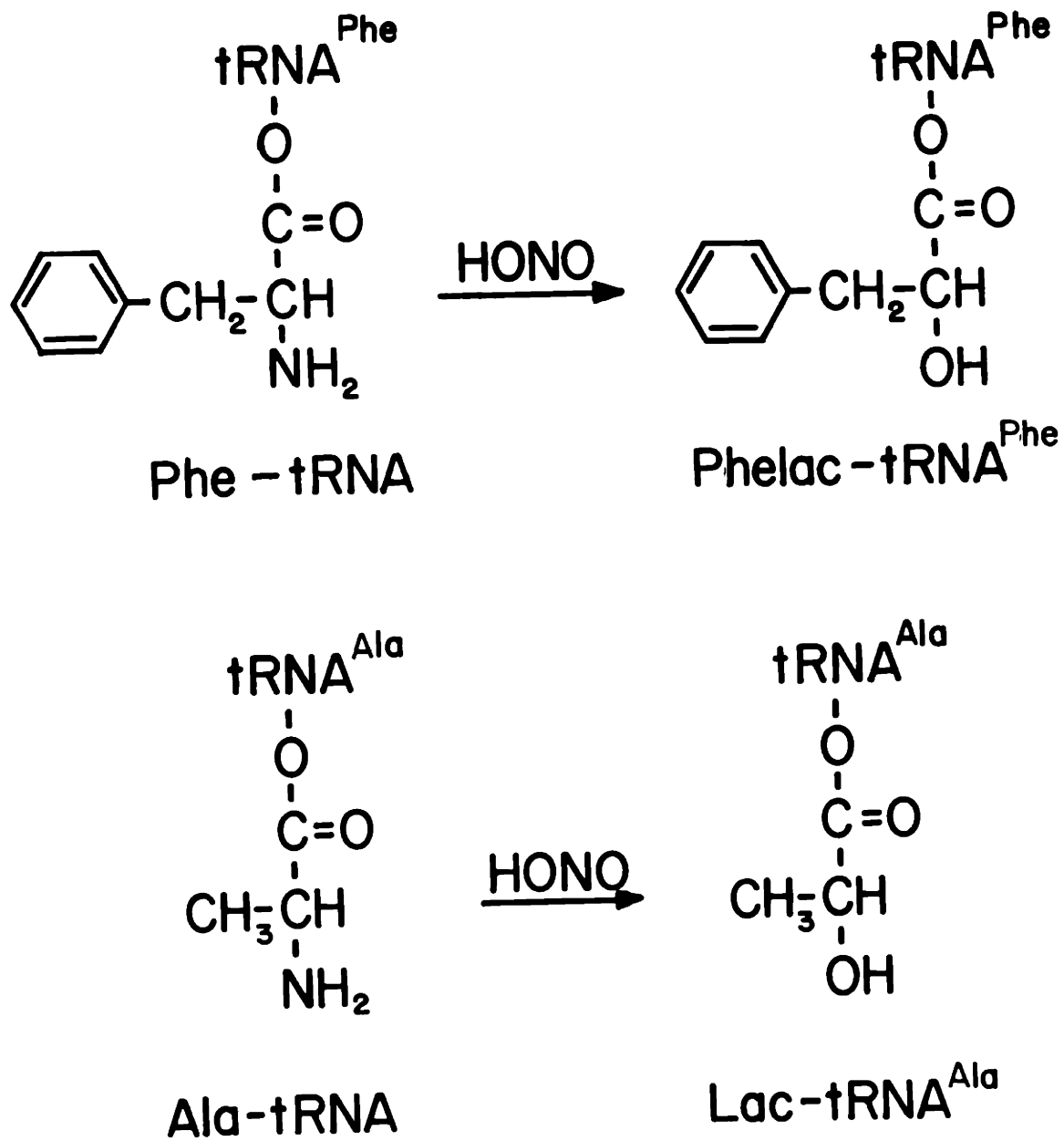


Figure 22

to its action in peptide bond formation.

ABBREVIATIONS

BSA -- bovine serum albumin

GMP-PCP -- 5'-guanylyl methylene diphosphonate
(β - γ methylene analog of GTP)

Lac -- L-lactic acid

Phe -- L-phenylalanine

Phelac -- L-(-)-3-phenyllactic acid

Phelac-Phe -- L-phenyllactyl-L-phenylalanine

PEP -- phosphoenol pyruvate, sodium salt

poly U -- polyuridylic acid, potassium salt

SDS -- sodium dodecyl sulfate

TCA -- trichloroacetic acid

tRNA^{Phe} -- transfer RNA specific for Phe

Other abbreviations are listed in the previous section.

MATERIALS

L-phenylalanine and L-(-)-3-phenyllactic acid were purchased from Aldrich Chemical Company. (^{14}C)L-phenylalanine (459 mC/mmol), uniformly labeled, was obtained from Amersham/Searle. L-alanine and DL-lactic acid were obtained from Sigma Chemical Company. GMP-PCP was purchased from Miles Laboratories. PEP was obtained from Sigma Chemical Company. Uridine-[5- ^3H] and poly U (number average molecular weight 2×10^6) were purchased from Schwartz BioResearch. Acid hydrolysate of casein (casamino acids) was obtained from Difco Laboratories. Bovine serum albumin, as a 30% w/v solution, was obtained from Armour Pharmaceutical Company.

Phosphocellulose (Whatman P-11) and Whatman chromatography paper were purchased from Reeve Angel. Silica gel G thin layer plates (Chromagram Sheet 6060) were obtained from Eastman. Nitrocellulose filter material was obtained from Millipore Corporation. Dowex 50W-X2 (AG 50WX2) was obtained from Bio-Rad Laboratories.

The dipeptide phenyllactyl-phenylalanine was obtained by nitrous acid treatment of phenylalanyl-phenylalanine (Sigma Chemical Company) (Hervé and Chapeville, 1965). Phenylalanyl-phenylalanine (10 mg) was dissolved in 1 ml glacial acetic acid. One ml H_2O and 1 ml NaNO_2 solution (saturated at 2°C) were added and the mixture incubated 1 hour at 25°C . Concentrated HCl was then added to lower

the pH to about 1, and the product was extracted with 3 ml ethyl acetate.

N-Formylmethionyl-alanine (fMet-Ala) and N-formylmethionyl-alanyl-serine (fMet-Ala-Ser) were prepared by formylating the appropriate peptides (purchased from Mann Research Laboratories) by the method of Sheehan and Yang (1958). This procedure is described in the previous section.

E. coli strains S26 and S26R1E(Su⁺) were obtained from A. Garen. *E. coli* W3110 was obtained from S. E. Luria. Phage R17 amB₂ was obtained from M. Capecchi.

Sources of other items are described in the previous section.

METHODS

Salt-washed ribosomes and S-100 were prepared from *E. coli* Q13 as described in the previous section.

Native ribosomal subunits with initiation factors intact were obtained by cold treatment of *E. coli* Q13 (Friedman, et al, 1969). A 15 liter culture was grown to mid-log phase in tryptone-yeast extract medium as described in the previous section, then chilled with ice and maintained at 3° to 7° C for 5 hours with aeration. Cells were harvested and washed, then lysed by alumina grinding and treated as described in the previous section through the second 33,000 rpm centrifugation. Ribosomes were pelleted by centrifugation for 20 hours at 50,000 rpm in a Spinco 65 rotor, through 6 ml 1 M sucrose containing 0.03 M NH₄Cl, 0.05 M Tris pH 7.4, 0.01 M magnesium acetate, and 0.006 M β-mercaptoethanol. Ribosome pellets were rinsed and resuspended in standard buffer. Aliquots were frozen in dry ice and stored at -20° C. The preparation was analyzed by zone sedimentation in a sucrose density gradient containing 0.01 M magnesium and found to consist of 30% 70S particles, 50% 50S, and 15% 30S.

RNA-free S-100 was prepared as described by Bretscher (1968). S-100 (50 ml) prepared as described in the previous section was treated with ribonuclease (0.5 μg) and 3 ml 0.2 M EDTA, pH 7.4, for 3 hours at 30° C. It was then

passed through a 1.8 cm × 20 cm phosphocellulose column equilibrated with 0.02 M Tris-HCl pH 7.4, 0.002 M β -mercaptoethanol and eluted with the same buffer. The first peak of absorption at 280 nm was collected and the protein precipitated by adding 0.56 g/ml ammonium sulfate. The precipitate was collected by centrifugation after standing overnight at 0° C, and redissolved in 5 ml 0.1 M Tris-HCl pH 8.6 plus 2 mM β -mercaptoethanol. This solution was passed through a 2.8 cm × 52 cm column of Sephadex G25 equilibrated with standard buffer, and eluted with standard buffer. The first A_{280} peak was collected (void volume), frozen in aliquots by dry ice and stored at -20° C.

Aminoacyl-tRNA Synthesis

The standard system for aminoacyl-tRNA synthesis contained, per ml: 0.1 M HEPES pH 7.4, 20 mM magnesium acetate, 7.5 mM ATP, 0.2 mM CTP, 20 mM creatine phosphate, 70 μ g creatine phosphokinase, 10 mM glutathione, S-100 (2 mg protein), 2 to 5 mg stripped tRNA, and amino acids. In addition, mixtures for fMet charging contained 0.6 mM folinic acid. Incubation was for 15 min. at 37° C. The mixture was then chilled on ice and the pH lowered to about 5 with acetic acid. Protein was extracted by shaking at 2° C with an equal volume of vacuum distilled, water saturated phenol, followed by centrifugation for

15 min. at 18,000 xg in a Servall SS-1 rotor at 2° C. RNA was precipitated from the aqueous phase by adding 0.1 volume 20% w/v potassium acetate, pH 5, and 2 volumes of cold ethanol, and was collected after 30 min. at -20° C by centrifugation.

(¹⁴C)Phe-tRNA was prepared in this standard system (but with Tris instead of HEPES), using 2 mg/ml *E. coli* K12 stripped tRNA (purchased from General Biochemicals) and 2.5 μ C/ml (¹⁴C)Phe, specific activity 459 mC/mmol. The ethanol precipitate was redissolved in 1 ml cold H₂O and passed through a 0.7 x 25 cm column of Sephadex G25 equilibrated with H₂O at 2° C. The first peak of 260 m μ absorbance was collected. The product contained 28 μ moles Phe per A₂₆₀ unit.

(¹⁴C) Phelac-tRNA was prepared from (¹⁴C)Phe-tRNA by nitrous acid deamination under conditions which were shown by Carbon (1965) to result in minimal alteration of the tRNA. Three mg (¹⁴C)Phe-tRNA was dissolved in 1.5 ml 0.25 M sodium acetate pH 4.3, 0.01 M magnesium acetate, 1 M NaNO₂, and incubated at room temperature on a Radiometer pH-stat at pH 4.3 for 30 min. RNA was then precipitated with ethanol at -20° C, the precipitate redissolved in water and reprecipitated, and the pellet washed with ethanol. Ethanol was removed under vacuum and the residue dissolved in water and stored at -20°C. The product

was analyzed by paper chromatography after alkaline hydrolysis, and found to contain less than 0.5% phenylalanine (see below).

A similar preparation was carried out using purified *E. coli* tRNA^{Phe 2} (78% pure) obtained from Oak Ridge National Laboratories. This preparation was used only for the T-factor binding experiments; all other experiments used unfractionated tRNA. Purified tRNA^{Phe} was charged with (¹⁴C)Phe of specific activity 214 mC/mmol and treated with nitrous acid as described above. The product was analyzed as follows. An aliquot was incubated in 1 M triethylamine (pH 12.5) at 37° C for 30 min., then subjected to paper chromatography (Whatman No. 1, solvent n-butanol/acetic acid/water [78:5:17]) and to silica gel thin layer chromatography in the same solvent. The paper chromatogram was scanned on a Vanguard strip counter and the silica gel plate was subjected to autoradiography on Kodak Royal Blue X-ray film. The results are shown in Figure 23. Non-radioactive carrier Phe and Phelac were located by means of their fluorescence in liquid nitrogen under ultraviolet excitation. Paper chromatography demonstrates that less than 0.1% of the radioactivity is phenylalanine. About 20% remains at the origin and most of the radioactivity moves with carrier Phelac. Thin layer chromatography reveals that most of the radioactivity cochromatographs with carrier

FIGURE 23: CHROMATOGRAPHIC ANALYSIS OF THE
PRODUCT OF NITROUS ACID TREATMENT
OF Phe-tRNA.

The procedure is described in the text.
(a) Paper chromatography of the triethyl-
amine-treated material. (b) Silica gel
chromatography.

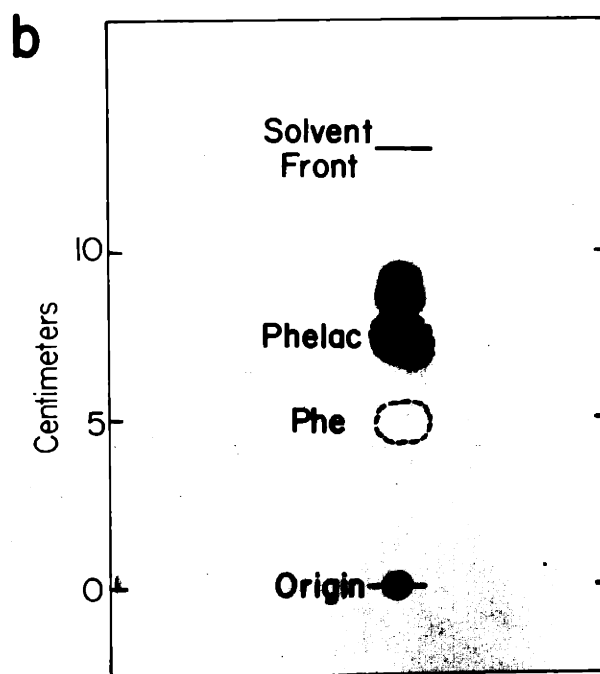
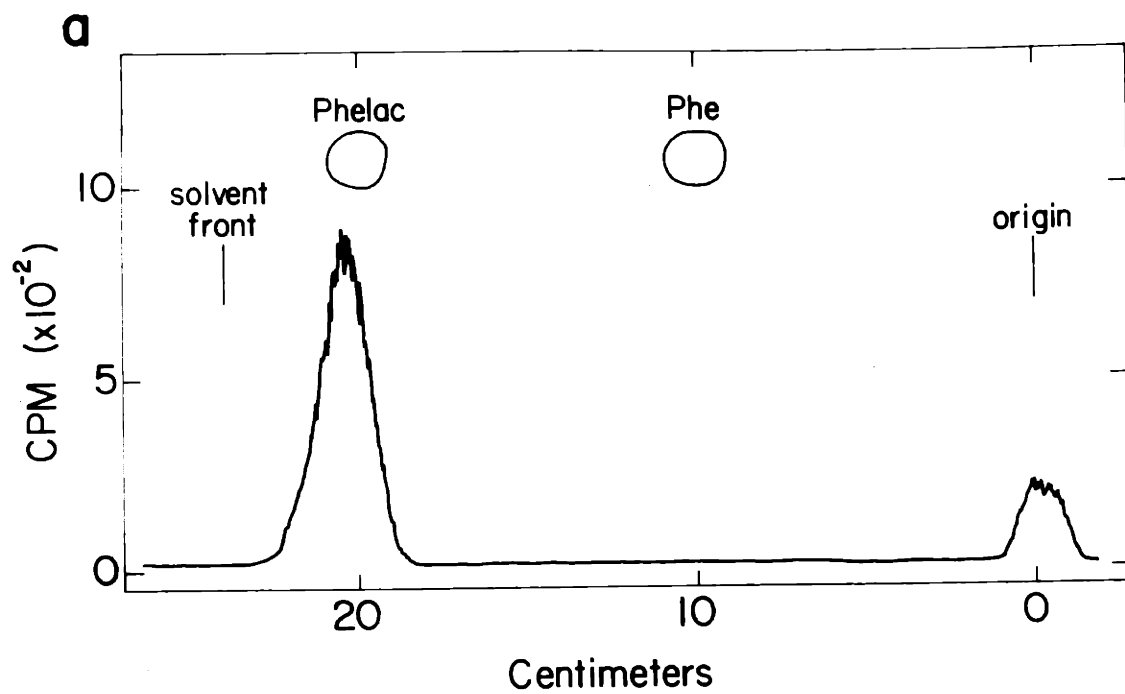


Figure 23

Phelac but there are two faster moving components. These may arise from rearrangements like those encountered in the nitrous acid deamination of puromycin. In that reaction the rearranged products comprised 90% of the yield. However, the presence of the p-methoxy group on the aminoacyl portion of puromycin would be expected to increase the amount of rearrangement observed (Roberts and Regan, 1951) so less rearrangement is expected in the deamination of Phe, and less is apparently observed. A significant portion of the radioactivity remains at the origin on silica gel also. This material may be unhydrolyzed Phelac-tRNA or some other rearrangement product. If it is unhydrolyzed tRNA it must contain less than 0.1% Phe, since Phe-tRNA is more labile in alkali than is Phelac-tRNA (Hervé and Chapeville, 1965).

In the above charging of purified tRNA^{Phe} a parallel incubation was carried out using tRNA which had previously been treated with nitrous acid in the standard way. Untreated tRNA accepted 643 $\mu\text{moles Phe/A}_{260}$ unit, while nitrous acid treated tRNA accepted 454 $\mu\text{moles/A}_{260}$ unit. This 31% reduction in the amino acid acceptance of the tRNA after nitrous acid treatment reveals that there is some damage to the tRNA during such treatment. The treatment of Hervé and Chapeville (1965) was reported to decrease the Phe acceptance of tRNA by 70 to 80%.

(¹⁴C) lactyl-tRNA was prepared from (¹⁴C) alanyl-tRNA in the same way. (¹⁴C)Ala-tRNA was prepared (using mixed tRNA) and treated with nitrous acid as described above. The product was treated with alkali and analyzed by paper chromatography as described above. The distribution of radioactivity is shown in Figure 24. The bulk of radioactivity cochromatographs with carrier lactic acid and there is no detectable alanine.

(³H)tRNA was prepared from *E. coli* W3110 labelled with Uridine-[5-³H]. A culture (500 ml) was grown to mid-log phase ($A_{550} = 1.0$) in a medium containing, per liter, 0.9 g NH₄Cl, 0.27 g MgSO₄, 0.03 g CaCl₂, 9 mg gelatin (Difco), 22 g glycerol, 13.5 g casamino acids, 1.05 g Na₂HPO₄, 0.45 g KH₂PO₄, and 2 mg (5 mC) uridine-[5-³H]. Cells were harvested by centrifugation, washed with a buffer containing 0.001 M Tris-HCl pH 7.4 and 0.01 M MgCl₂, and resuspended in 1 ml of the same buffer. RNA was extracted by the method of von Ehrenstein (1968). The suspension was extracted with 1 ml phenol at 2° C, the aqueous phase was removed and the phenol reextracted with 1 ml buffer. RNA was precipitated from the combined aqueous layers with 0.1 volume 20% potassium acetate (pH 5) and 2 volumes of cold ethanol. Amino acids were stripped from the tRNA by redissolving the pellet in 1.5 ml 1 M Tris-HCl pH 8.6, which had been autoclaved to destroy

FIGURE 24: CHROMATOGRAPHIC ANALYSIS OF THE
PRODUCT OF NITROUS ACID TREATMENT
OF Ala-tRNA.

(^{14}C)Ala-tRNA was prepared and treated with nitrous acid as described in the text. An aliquot of the product was hydrolyzed with 1 M triethylamine (37° C, 30 min.) and analyzed by paper chromatography (Whatman No. 1; solvent n-butanol/acetic acid/water [78:5:17]). Radioactivity was located on a Vanguard gas flow strip counter, and carrier lactic acid was located by spraying with 0.04% bromocresol green in 95% ethanol.

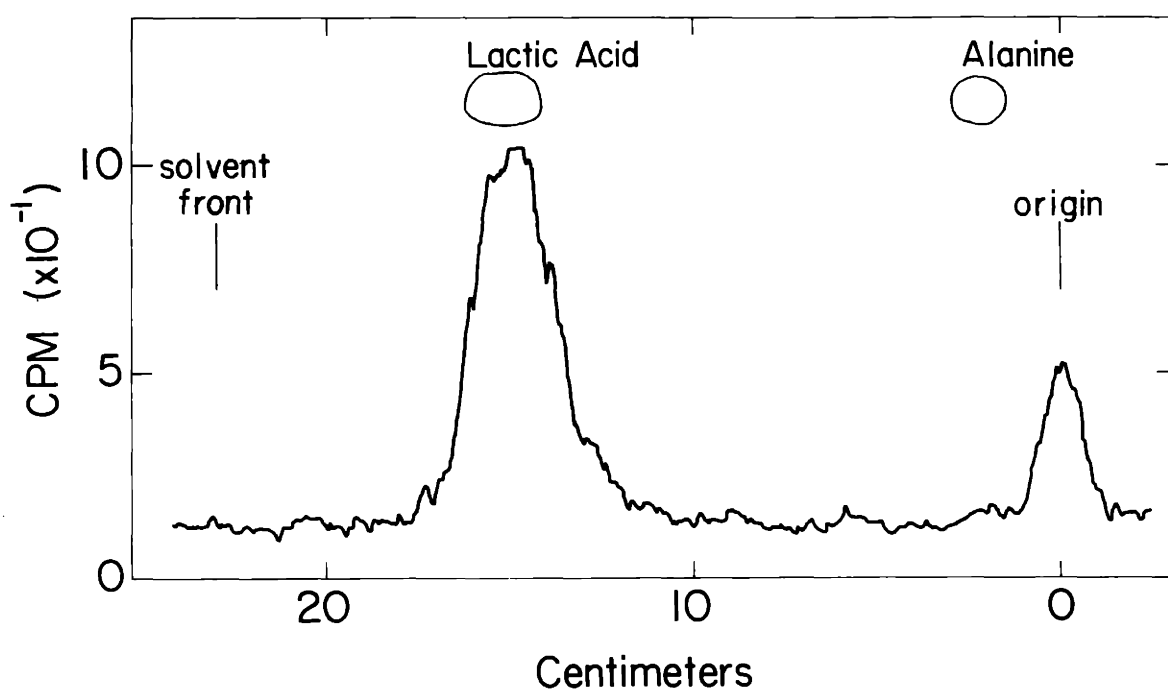


Figure 24

ribonuclease, and incubating for 90 min. at 37° C. RNA was again precipitated with ethanol, and the ethanol removed under vacuum.

tRNA was then purified by the method of Avital and Elson (1969). The dried RNA pellet was finely suspended in 0.4 ml 2 M LiCl, 0.1 M potassium acetate pH 5.0 at 0° C and shaken vigorously on a Vortex mixer for 20 min. at 2° C. It was then allowed to stand in an ice bath for 15 min. and the insoluble material removed by centrifugation. The pellet was extracted with another 0.2 ml aliquot of 2 M LiCl, 0.1 M potassium acetate, pH 5.0, and the combined supernatants were dialyzed for 2.5 hours against 5 × 1 liter 10^{-6} M magnesium acetate. Ammonium sulfate (0.315 g/ml) was added, the suspension stirred in an ice bath for 25 min., and the precipitate removed by centrifugation. To the supernatant was added 1.1 g ammonium sulfate for each g added previously and the mixture was again stirred and the tRNA precipitate removed by centrifugation. The pellet was dissolved in H₂O and dialyzed overnight against 2 × 1 liter 10^{-6} M magnesium acetate. The yield was 3.5 mg tRNA containing 279 μ C tritium.

This [³H]tRNA was charged with (¹⁴C) phenylalanine (459 mC/mole) and treated with nitrous acid as described above. The final (¹⁴C)Phelac-(³H)tRNA contained, per A₂₆₀

unit of RNA, 2.42 μC ^3H and 0.0179 μC ^{14}C . Less than 0.3% of the ^{14}C was found to be Phe.

Non-radioactive tRNA was also prepared from *E. coli* B (purchased from General Biochemicals) by the same method. This tRNA was used in the R17 RNA experiments since tRNA purchased from General Biochemicals was found to be deficient in asparagine accepting activity.

(^{35}S) fMet-tRNA was prepared in the standard way using (^{35}S) methionine prepared as described in the previous section. It was then freed of aminoacyl-tRNA by treatment with 0.02 M CuSO_4 , 0.2 M sodium acetate, pH 5.5, for 60 min. at 37° C (Schofield and Zamecnik, 1968).

R17 RNA Preparation

Bacteriophage R17 amB₂ was grown on *E. coli* S26R1E (Su^+). A 10 liter culture growing exponentially in the tryptone-yeast extract medium described in the previous section was inoculated at $A_{450} = 0.7$ (5×10^8 cells/ml) with phage at a multiplicity of 10, in terms of plaque forming units (titered on *E. coli* S26R1E). Twenty ml 1 M CaCl_2 was also added. Aeration was continued for 4 hours during which time the A_{450} rose to 2.0 then dropped to 0.6 (Titer-- 1.3×10^{12} pfu/ml). Chloroform (100 ml) and DNase (0.2 mg) were added, and after 10 min. at 37° C, 250 ml 0.1 M EDTA and 3465 g ammonium sulfate were added. The carboy was shaken until the ammonium sulfate was dissolved,

then stored overnight at 0° C. Phage were then purified by the method of Gesteland and Boedtker (1964). The precipitate was collected by centrifugation, using a Servall continuous flow apparatus, then resuspended in 100 ml of a buffer containing 0.01 M Tris-HCl pH 7.4, 0.05 M EDTA, 0.1 M NaCl. The solution was clarified by centrifugation in a Servall SS-1 rotor for 10 min. at 10,000 xg. Phage particles were precipitated by adding one third volume cold methanol, and collected by centrifugation after 2.5 hours at -20° C. The pellet was resuspended in 35 ml 0.05 M Tris-HCl pH 7.4, 0.005 M EDTA, 0.1 M NaCl, and again clarified by centrifugation. Phage were then pelleted by centrifugation for 1 hour at 50,000 rpm in a Servall 65 rotor and resuspended in 0.05 M Tris-HCl pH 7.4, 0.001 M EDTA, 0.1 M NaCl.

Phage were further purified by banding in CsCl. Per ml of phage suspension, 0.659 g CsCl was added. After centrifugation for 22 hours at 50,000 rpm in a Spinco 65 rotor at 2° C, the major opalescent band was removed with a syringe and dialyzed overnight against 4 × 1 liter 0.05 M Tris-HCl, pH 7.4, 0.001 M EDTA, 0.1 M NaCl. Yield: 164 mg, determined spectrophotometrically using the extinction coefficient of Gesteland and Boedtker (1964). Stored as a suspension in the above buffer at 2° C. The final product contained 0.28% wild type revertants [titer on

E. coli S26 (Su^-)/titer on S26R1E (Su^+)].

RNA was extracted from the phage by the method of Oda and Joklik (1967). Sixty mg purified phage particles were suspended in 15 ml 0.05 M sodium acetate, 0.01 M EDTA, pH 5.1. 150 mg sodium dodecyl sulfate (SDS) was added and the solution made 0.5 M in NaClO_4 . It was then shaken for 2 min. with an equal volume of chloroform/isoamyl alcohol (24:1), chilled, and the phases separated by centrifugation. The organic phase was re-extracted with 17 ml 0.05 M sodium acetate, pH 5.1, 0.01 M EDTA, 0.5 M NaClO_4 , 1% SDS. The combined aqueous phases were shaken with 34 ml chloroform/isoamyl alcohol (24:1). RNA was precipitated from the final aqueous phase with one-fifth volume 1 M NaCl + 2 volumes ethanol, then dialyzed against 0.01 M Tris-HCl pH 7.4, 0.001 M magnesium acetate in dialysis tubing which was previously treated with diethyl pyrocarbonate to inactivate ribonuclease (Solymosy, et al, 1968). Sucrose gradient analysis of the product revealed a single component sedimenting near 30S, with less than 10% smaller material. Yield: 15 mg, determined spectrophotometrically using the extinction coefficient of Gesteland and Boedtker (1964).

Poly U-Directed System

The incorporation system -- In general the incorporation system contained 0.11 M Tris-HCl, pH 7.4; 0.096 M NH_4Cl ; 0.016 M magnesium acetate; 0.5 mM GTP; 4 mM PEP; 0.89 mg/ml S-100 protein; 2.6 A_{260} units/ml ribosomes; 10 A_{260} units/ml (^{14}C)Phelac-tRNA (28 μmoles Phelac/ A_{260} unit, at 459 mC/mmmole); and 24 $\mu\text{g/ml}$ poly U. Components were generally mixed on ice in the order shown. The incorporation was initiated by transfer to 30° C, at which temperature they were incubated for time periods listed in the figure legends.

The assay -- To each sample was added 0.5 volume of a solution of pancreatic ribonuclease (0.5' mg/ml) in 0.1 M EDTA, pH 7.4. They were then incubated for 15 min. at 30° C. Cold 10% TCA (40 times the original sample volume) containing 0.5% w/v casamino acids was added and the samples passed through millipore type HAWP filters. The filters were washed three times with 3 ml aliquots of 5% TCA containing 0.25% w/v casamino acids, affixed to aluminum planchets by means of a drop of 1% ovalbumin, and dried under an infrared lamp. They were then counted at an efficiency of 23% in a Nuclear Chicago low background gas flow counter.

This assay is based on the ability of the EDTA-RNase treatment to degrade all tRNA both ribosome bound and

unbound, so that all unincorporated Phelac-tRNA is rendered TCA soluble. The validity of the assay is indicated by the data in Figure 25. Phelac-tRNA was incubated with ribosomes and poly U, then digested for 5 min. at 30° C with various amounts of RNase, with and without EDTA. The incubation allows binding of most of the phelac tRNA to ribosomes, as indicated by the fact that 70% of the phelac is protected from subsequent attack by 0.1 µg/ml RNase, only if both ribosomes and poly U were included in the first incubation (Pestka, 1968). In the presence of EDTA, 10 µg/ml RNase renders 99% of the Phelac acid soluble. In the assay used in the experiments described in this section, the final concentration of ribonuclease is 167 µg/ml, and the digestion is carried out for 15 min. Thus the assay is sufficient to solubilize more than 99% of the Phelac-tRNA, whether it is bound to ribosomes or free.

R17 RNA-Directed System

The incorporation system -- The incubation mixture (1 ml) contained 0.05 M Tris-HCl, pH 7.4, 0.007 M magnesium acetate, 0.06 M NH₄Cl, 0.2 mM GTP, 5.2 mM PEP, 6 mM β-mercaptoethanol, 8.0 A₂₆₀ units/ml R17 amB₂ RNA, 50 A₂₆₀ units/ml ribosomes (not salt washed, consisting of 60% native subunits), 0.32 mg/ml RNA-free S-100, 4.0 A₂₆₀ units/ml tRNA charged with (³⁵S)fMet (5.0 × 10⁴ dpm/A₂₆₀ units) and 20 A₂₆₀ units/ml tRNA charged with

FIGURE 25: RIBONUCLEASE DIGESTION OF PHELAC-tRNA.

Phelac-tRNA was bound to ribosomes then treated with various amounts of ribonuclease with and without EDTA. The complete binding system contained 0.1 M Tris-HCl, pH 7.4; 0.09 M NH_4Cl ; 0.015 M magnesium acetate; 1.0 A_{260} unit/ml (^{14}C)phelac-tRNA (28 μmoles phelac/ml at 459 mC/mmmole); 24 $\mu\text{g/ml}$ poly U; and 26 A_{260} units/ml ribosomes. Samples were also incubated without poly U or without ribosomes, as noted in the figure. Samples (0.1 ml) were incubated 10 min. at 30° C, then chilled on ice. EDTA (0.05 ml, 0.1 M) was added to the samples so indicated in the figure, and the appropriate amount of ribonuclease (in 1 μl) was added. Samples were again incubated for 5 min. at 30° C, then carrier BSA (0.125 mg) and 2 ml cold 10% TCA containing 0.5% casamino acids were added. The samples were millipore filtered and counted as described in the text.

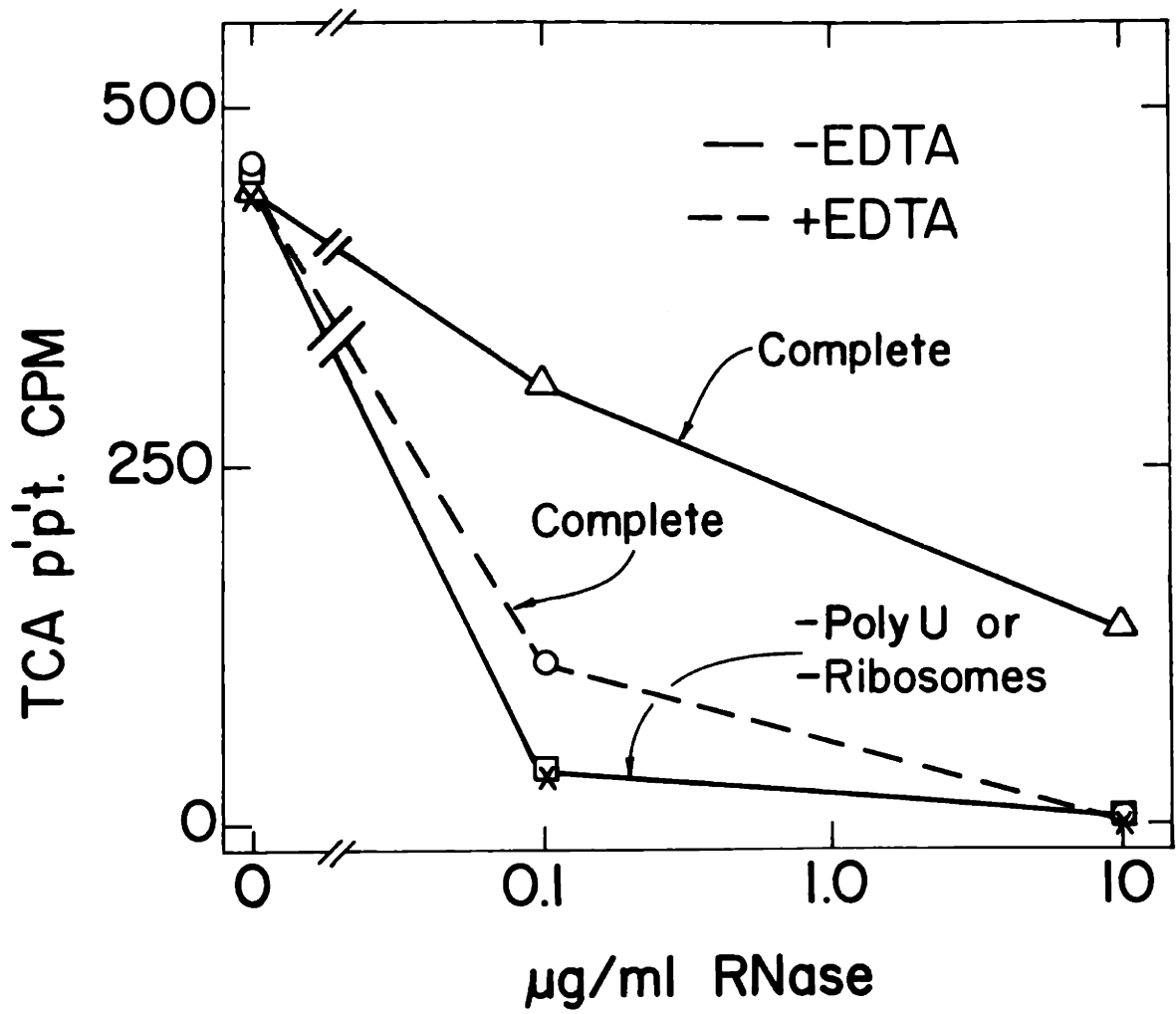


Figure 25

unlabelled amino acids and α -hydroxyacids as described below. The mixture was incubated at 34° C for 30 min., then the TCA soluble, N-blocked peptides were isolated according to the procedure of Capecchi (1967). To 1 ml incubation mixture (chilled) was added 4 ml cold 5% w/w TCA, and the precipitate was removed by centrifugation. The supernatant was extracted by shaking with 5 ml of a mixture of m-cresol and ethyl acetate (0.7 ml:0.3ml). The phases were separated by centrifugation at room temperature, and the upper (organic) phase removed. To the organic phase was added 1 ml H₂O and 10 ml ether, and after shaking and centrifugation the ether phase was removed and the aqueous phase washed with 10 ml ether. The last traces of ether were removed in a stream of air and the aqueous phase passed through a column (0.5 cm \times 2 cm) of Dowex 50-X2, equilibrated with H₂O. The material was eluted with H₂O and lyophilized.

The hexapeptide was further purified by electrophoresis on paper (Whatmann 3MM) in a buffer composed of 14 ml pyridine + 12.5 ml glacial acetic acid per liter (pH 4.8), at 28 v/cm for 5 hours. The hexapeptide region was eluted. An aliquot of this material was treated with 1 M triethylamine (pH about 12.5) at 35° C for 20 min. Triethylamine treated and untreated samples were then subjected to electrophoresis at 4° C on a cellulose thin layer (E. Merck)

in the above pyridine-acetate buffer, pH 4.8, for 105 min. at 30 v/cm. Radioactivity was located by autoradiography on Kodak Royal Blue X-ray film.

Preparation of α -hydroxyacyl tRNAs -- tRNA was prepared from *E. coli* B according to the procedure of Avital and Elson (1969) described above. Five mg tRNA was charged with alanine or phenylalanine in the standard incubation mixture using S-100 (2 mg/ml protein) chromatographed on Sephadex G25 immediately before use, and 10 mM phenylalanine or alanine. After incubation at 30° C for 30 min., tRNA was recovered by phenol extraction and ethanol precipitation. The RNA was dissolved in 2 ml 0.25 M sodium acetate, pH 4.3, 0.01 M magnesium acetate, 1 M NaNO₂, and incubated at room temperature (23° C) on a pH-stat at pH 4.3 for 30 min. RNA was precipitated with ethanol and charged with a mixture of amino acids described in the text. RNA-free S-100 was used in this second charging incubation, which was otherwise as described above.

The products of the nitrous acid treatment of alanyl- and phenylalanyl-tRNA are lactyl- and phenyllactyl-tRNA, respectively. This was verified by chromatography of alkaline digests of similar preparations made with the (¹⁴C) amino acids described above.

Interaction between T factor and Phelac-tRNA

(¹⁴C)Phe-tRNA and Phelac-tRNA were prepared from

purified tRNA^{Phe} as described above. Phe-tRNA was made from tRNA which had previously been treated with nitrous acid as described above. It contained 454 $\mu\mu$ moles Phe/A₂₆₀ unit at a specific activity of 214 mC/mmole. Phelac-tRNA was made from tRNA which had not previously been treated with nitrous acid, and it contained 643 $\mu\mu$ moles of Phelac/A₂₆₀ unit at the same specific activity.

These experiments were performed with Dr. H. Weissbach, who provided all other components. T_U and T_S were purified preparations (Ertel, et al, 1968). Ribosomes (NH₄Cl washed) were prepared as described by Ertel, et al, (1968).

Ternary complex formation was determined by the millipore binding technique of Ertel, et al, (1968b). [T_U-GDP] was converted to [T_U-GTP] by a preliminary incubation for 15 min. at 37° C in a mixture containing, per 0.2 ml aliquot, 0.05 M Tris-HCl pH 7.4, 0.01 M MgCl₂, 0.05 M NH₄Cl, 3.7 mM PEP, 50 μ g/ml pyruvate kinase, 500 $\mu\mu$ moles [³H]-GTP, and 45 units purified [T_U-GDP]. (A unit of T_U is the quantity which binds 1 $\mu\mu$ mole of [³H]GDP.) Samples were then chilled on ice and Phelac-tRNA^{Phe} or Phe-tRNA was added. After 5 min. incubation at 0° C each sample was diluted with 2 ml cold buffer (0.05 M Tris-HCl, pH 7.4, 0.05 M NH₄Cl, 0.01 M MgCl₂) and passed through a millipore filter. The filter was washed thoroughly with cold buffer, dissolved in 10 ml Bray's solution and counted in a liquid

scintillation spectrometer. The difference between the amount of [^3H]GTP bound to the filter in the presence and absence of tRNA is the amount of ternary complex [T_U -GTP-tRNA] formed.

Ribosome binding was determined according to the method of Ertel, et al, (1968). The complete incubation mixture contained, in 0.05 ml, 0.05 M Tris-HCl, pH 7.4, 0.01 M MgCl_2 , 0.08 M NH_4Cl , 0.08 M KCl, 0.001 M dithiothreitol (DTT), 1.34 A_{260} units of NH_4Cl -washed ribosomes, 30 units T_U , 60 units T_S , 3 μg poly U, 1 μmole GTP, and (^{14}C)Phe-lac-tRNA or Phe-tRNA. After incubation for the appropriate time at 20° C each sample was diluted with 2 ml cold buffer (0.05 M Tris-HCl, pH 7.4, 0.01 M MgCl_2 , 0.08 M NH_4Cl , 0.08 M KCl, 0.001 M DTT), and passed through a millipore filter which was then thoroughly washed with buffer, dissolved in 10 ml Bray's solution, and counted on a liquid scintillation spectrometer.

Double Label Counting

Simultaneous determination of ^3H and ^{14}C in a single sample was done in a Nuclear Chicago liquid scintillation spectrometer using the channels ratio quench correction method described in a Nuclear Chicago Technical Bulletin by R. W. Hendler. Spillover and efficiency corrections were determined using a set of quenched standards in the same scintillator (Bray's solution) used for the samples. The

radioactive standards were ^{14}C and ^3H labelled toluene (Packard) quenched with various amounts of chloroform (Experiment I in Table 10) or 10% aqueous sucrose (Experiment II in Table 10).

RESULTS

The Poly U-Directed System

Hervé and Chapeville (1965) have synthesized phenyl-lactyl-tRNA by treating phenylalanyl-tRNA with nitrous acid, and have examined its behavior in an *in vitro* system containing polyuridylic acid and other components which are required for polyphenylalanine formation. They observed binding of Phelac-tRNA to ribosomes and its incorporation into TCA precipitable material. They concluded, however, that labelled phenyllactic acid residues were incorporated only into "N-terminal" positions of a polypeptide composed of unlabelled phenylalanine which was present as a contaminant in their supernatant preparation. Thus according to their interpretation they did not observe the functioning of Phelac-tRNA as a peptidyl transferase acceptor but only as a chain initiator.

The observation that ψ hydroxypuromycin can serve as a peptidyl transferase acceptor, and that a compound derived from puromycin by nitrous acid treatment behaved similarly, suggested that it might be possible on further examination to observe the incorporation of phenyllactic acid into internal positions in a polymer, through peptidyl transferase-catalyzed ester bond formation. This phenomenon was observed. These results

contrast in many ways with those of Hervé and Chapeville, and possible explanations of the differences will be discussed below.

The Incorporation of Phelac

When (^{14}C)phenyllactyl-tRNA is provided in an *in vitro* system containing all of the components necessary for polypeptide synthesis, including ribosomes, S-100 protein, polyuridylic acid and GTP, phenyllactic acid is incorporated into a TCA precipitable product. The incorporation is assayed by precipitation with cold trichloroacetic acid after treatment with ribonuclease and EDTA under conditions where all tRNA, both ribosome bound and unbound, is rendered acid soluble. Figure 26 shows the time course of the incorporation.

The validity of this assay was demonstrated in the Methods section. However the incorporation can also be observed by means of an assay which does not depend on digestion of tRNA with ribonuclease. The points marked "Hot TCA" in Figure 27 were obtained by heating the precipitate to 90°C for 15 min. in 5% TCA. Incorporation assayed in this way is similar to that assayed by the RNase-cold TCA procedure ("Cold TCA" in Figure 27). This hot TCA treatment destroys RNA (Schneider, 1945).

FIGURE 26: TIME COURSE OF INCORPORATION OF
PHENYLLACTIC ACID INTO TCA PRECIPITATE.

The complete reaction mixture (0.6 ml) was as described in Methods. Similar aliquots were incubated without poly U or without S-100. After various incubation periods at 30° C, 0.05 ml aliquots were pipetted into tubes containing 0.025 ml of 0.1 M EDTA with 12.5 µg RNase. The RNase digestion was carried out for 15 min. at 30° C, then the tubes were chilled and 0.05 ml 2.5 mg/ml BSA and 2 ml cold 10% TCA containing 0.5% w/v casamino acids were added. The samples were millipore filtered and counted on a low background gas flow counter as described in Methods.

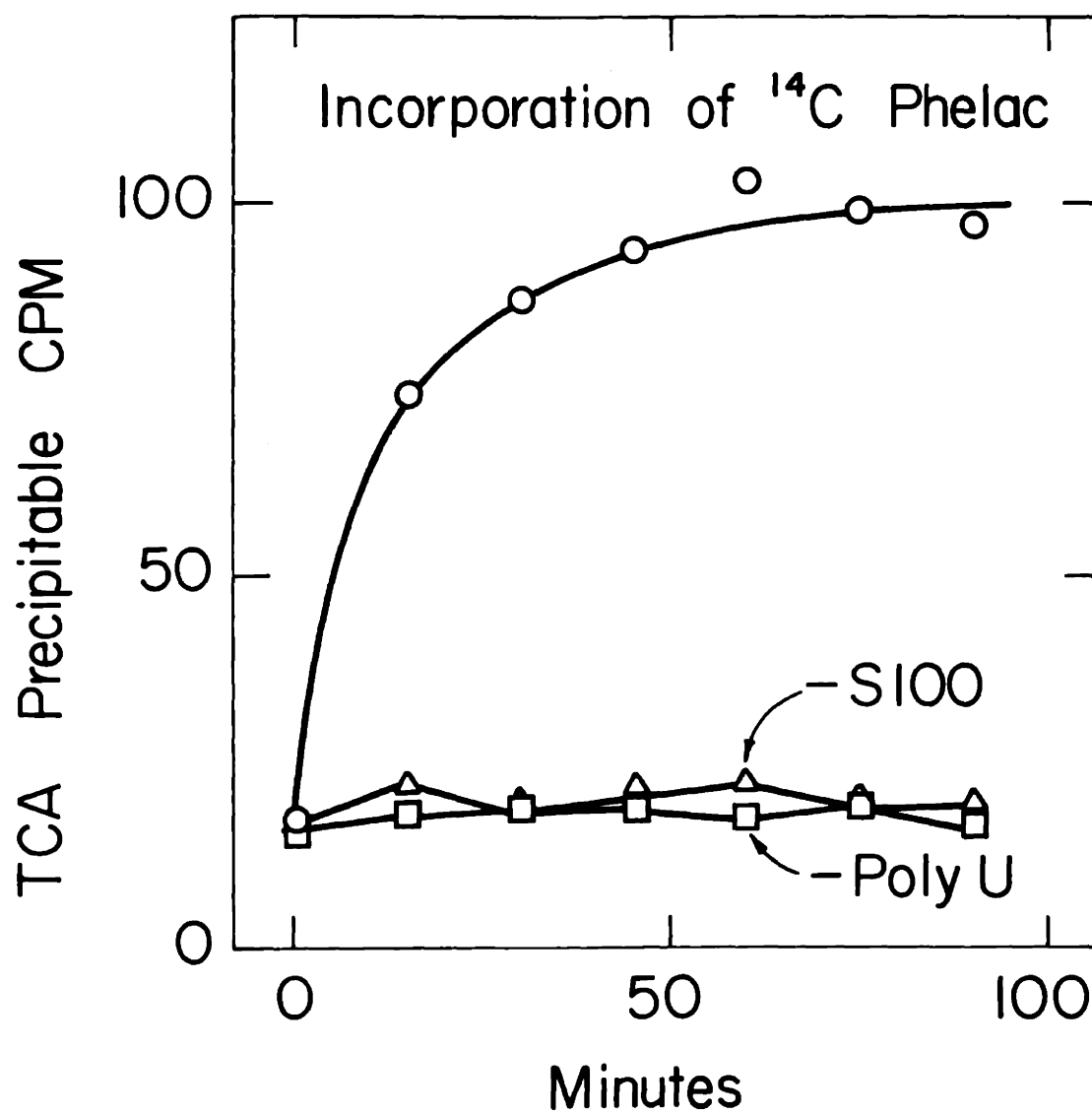


Figure 26

FIGURE 27: INCORPORATION OF PHELAC MEASURED BY
HOT AND COLD TCA PROCEDURES.

Incubation procedure was as described in Figure 26. The samples were assayed in two ways:

- (1) The usual RNase-EDTA treatment described in Methods, followed by addition of 2 ml cold 10% TCA containing 0.5% w/v casamino acids ("Cold TCA");
- (2) Same as (1) but followed by heating to 90° C for 15 min. ("Hot TCA"). All samples were millipore filtered and counted as described in Methods.

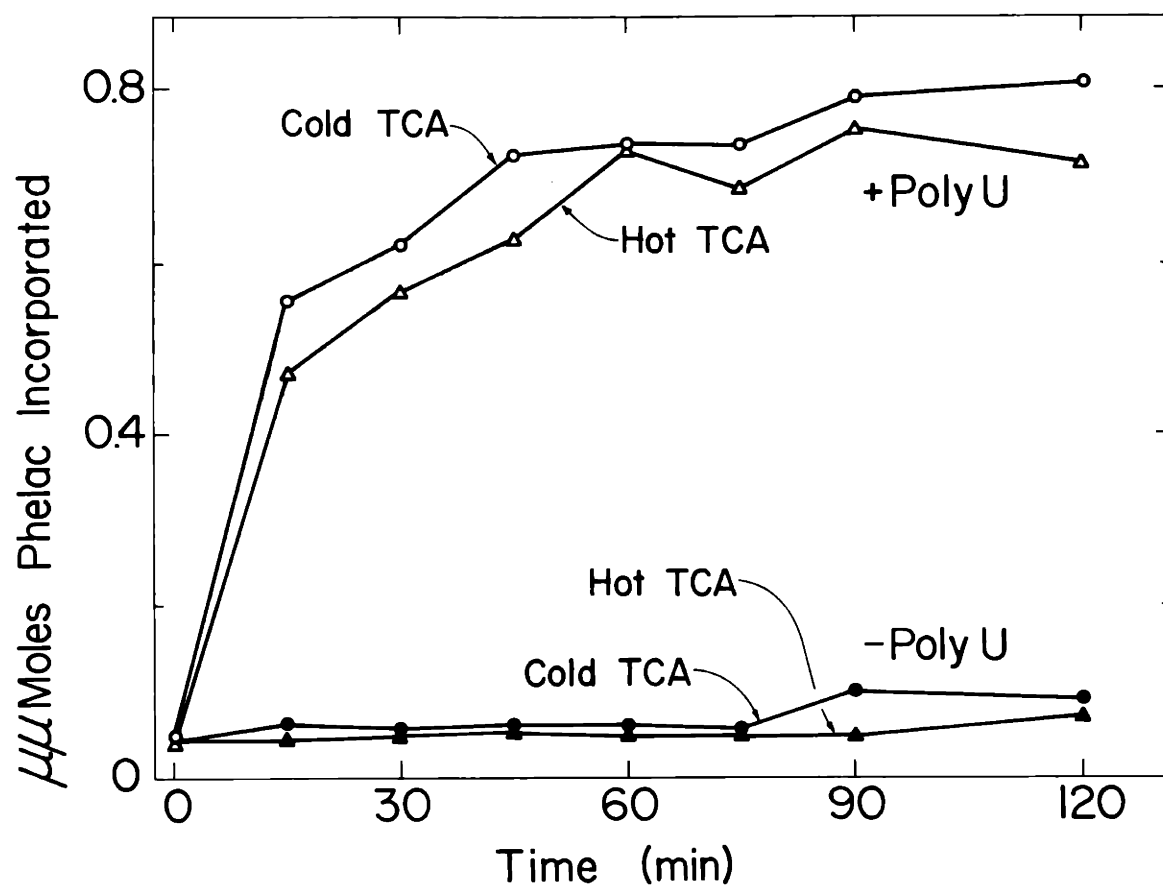


Figure 27

The material incorporated into the acid precipitate is shown to be phenyllactic acid by the analysis shown in Figure 28. The TCA precipitate was hydrolyzed in 11.6 N HCl at 110°C for 18 hours, then subjected to paper chromatography. Essentially all of the radioactivity travels with phenyllactic acid. There is no detectable (less than 1%) radioactive phenylalanine.

The appearance of acid insoluble material can be interpreted as a polymerization of residues by analog with the properties of polypeptide synthesizing systems. This interpretation is supported by the observation (Figure 29) that the incorporation product is destroyed by treatment with α -chymotrypsin. This enzyme is able to hydrolyze both ester and amide linkages (Dixon and Webb, 1964).

The incorporation of phelac shown in Figure 26 requires the presence of poly U and S-100. In addition the incorporation requires ribosomes (Figure 30) and GTP (Table 5). The data in Table 5 show that the GTP requirement cannot be satisfied by the β - γ methylene analog of GTP, GMP-PCP, and the analog inhibits the incorporation in the presence of low concentrations of GTP. GMP-PCP is active in the enzymatic binding of aminoacyl-tRNA to ribosomes (Haenni and Lucas-Lenard, 1968) but does not support amino acid polymerization

FIGURE 28 : CHARACTERIZATION OF THE INCORPORATED RADIOACTIVITY.

Incorporation was carried out as described in Methods. Two aliquots of 0.5 ml were treated in parallel, one incubated with the complete system and the other without poly U. After 60 min. incubation at 30° C, 0.1 ml 0.2 M EDTA and 0.1 ml 1 mg/ml RNase were added and the samples were incubated for 15 min. at 30° C. Carrier BSA (2 μ l 30% w/v) and 2 ml cold 10% TCA containing 0.5% w/v casamino acids were added. The precipitate was removed by centrifugation and washed twice with 5 ml aliquots of cold 5% TCA. TCA was removed in two hours on an oil vacuum pump, and the residue taken up in 0.5 ml conc. HCl. The samples were sealed in pyrex tubes and incubated 18 hours in a refluxing toluene bath (110° C). They were then dried on a Büchi flash evaporator, taken up in 0.1 ml H₂O, and spotted on Whatman #1 paper along with 20 μ g each of carrier phenylalanine and phenyllactic acid. Chromatography (descending) was carried out in a solvent system composed of n-butanol/acetic acid/water (78:5:17). The carrier phe and phelac spots were located under an ultraviolet lamp by their fluorescence in liquid nitrogen. The chromatograms were counted on a Vanguard gas flow strip counter.

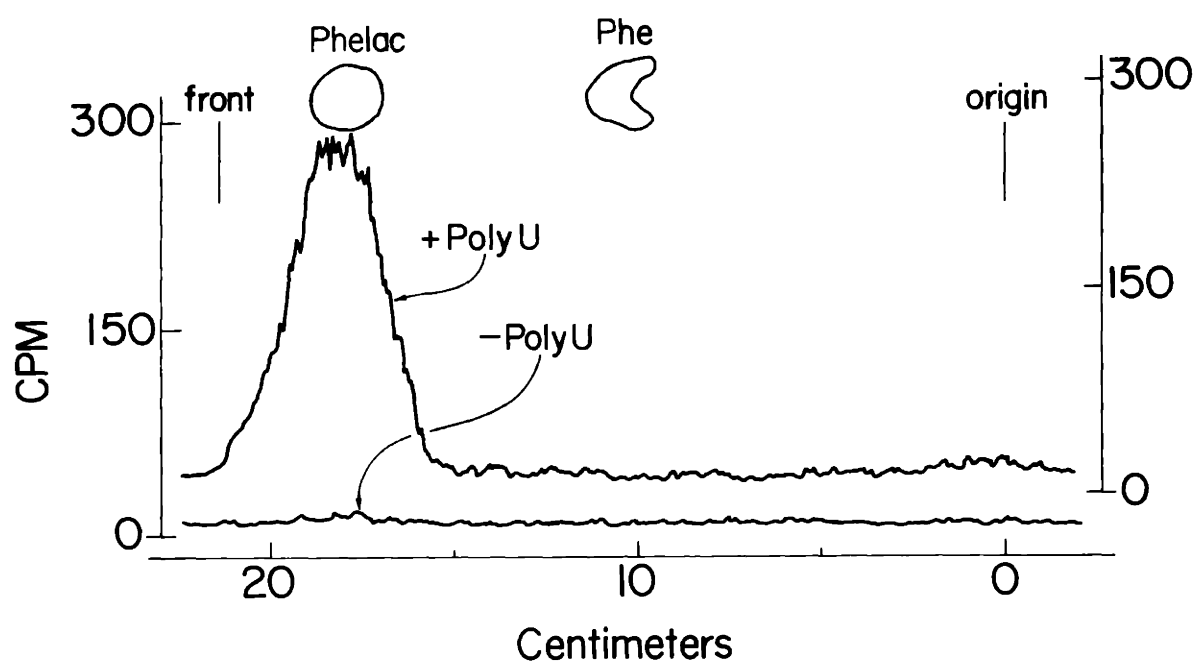


Figure 28

FIGURE 29: DEGRADATION OF THE TCA PRECIPITABLE
PRODUCT BY CHYMOTRYPSIN.

Incorporation from phelac-tRNA was carried out as described in Methods in a volume of 0.5 ml. After 60 min. at 30° C, 0.125 ml of each of 0.2 M EDTA and 1 mg/ml RNase were added and the sample again incubated at 30° C for 15 min. The pH was adjusted to 7.5 with ammonium hydroxide and 0.1 mg α -chymotrypsin was added in 0.1 ml 0.001 M HCl. At appropriate times after addition of chymotrypsin 0.1 ml aliquots were pipetted into tubes containing 2 ml cold 10% TCA plus 0.5% casamino acids. Carrier BSA was added and the samples filtered and counted as described in Methods.

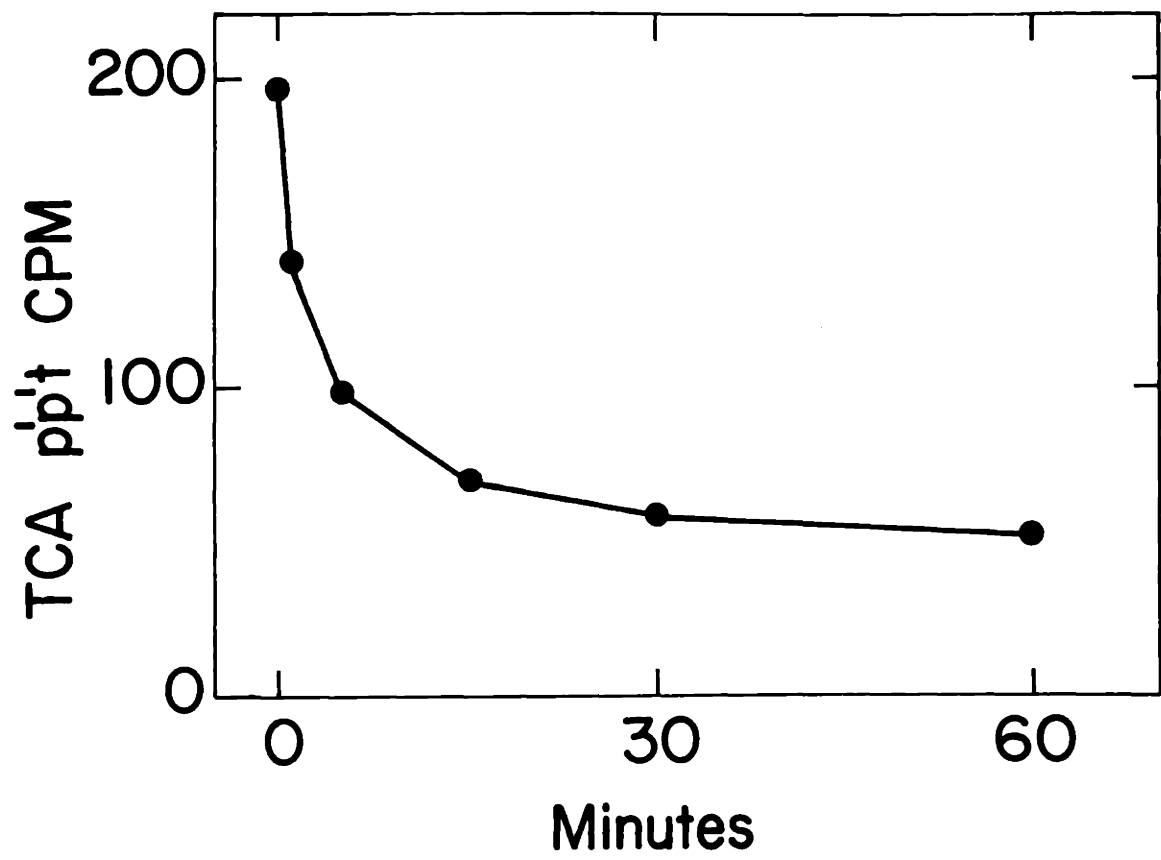


Figure 29

FIGURE 30: RIBOSOME DEPENDENCE OF THE PHELAC
INCORPORATION.

The incubation system was as described in Methods except the concentration of phelac-tRNA was twice the normal concentration. Aliquots (0.05 ml) were incubated with various amounts of ribosomes, with and without poly U. After 60 min. at 30° C the samples were treated with EDTA and RNase, precipitated with TCA, and counted as described in Methods. The units of the abscissa, which is graduated logarithmically to allow representation of a broad concentration range, are A_{260} units of ribosomes in each 0.05 ml incubation aliquot. The normal concentration, used in all the experiments described here, is 0.13 A_{260} units per 0.05 ml incubation mixture.

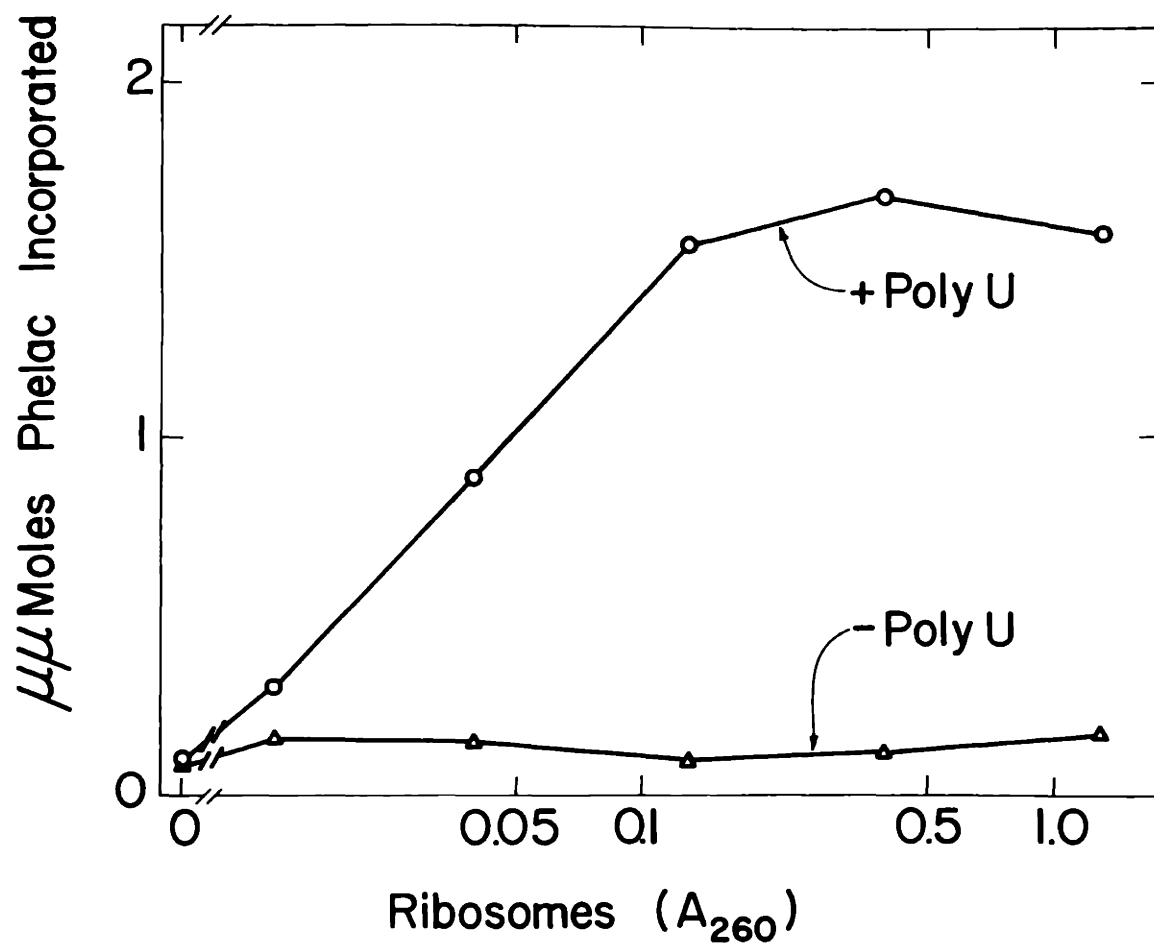


Figure 30

TABLE 5

DEPENDENCE OF PHELAC INCORPORATION ON GTP

	μ MOLES PHELAC INCORPORATED	
	<u>+ Poly U</u>	<u>- Poly U</u>
EXPERIMENT I		
Complete (plus PEP) 0.5 mM GTP	0.57	0.05
Minus GTP	0.44	0.05
Minus GTP plus 0.5 mM GMP-PCP	0.19	0.05
Minus GTP plus 1.2 mM GMP-PCP	0.13	0.05
EXPERIMENT II		
Complete (minus PEP) 0.5 mM GTP	0.64	0.11
Minus GTP	0.10	0.08
Minus GTP plus 0.5 mM GMP-PCP	0.10	0.08

The complete system in experiment I was as described in Methods. The complete system in experiment II was identical, but without phosphoenol pyruvate. Samples (0.05 ml) with and without poly U were incubated for 45 min. at 30° C, then assayed by the RNase-cold TCA procedure described in Methods.

because the γ -phosphate cannot be removed.

Thus the incorporation of phenyllactic acid requires the presence of all of the components which are required for polyphenylalanine formation in similar systems (Nirenberg and Matthaei, 1951). These requirements suggest that the observed incorporation is functionally related to polypeptide synthesis.

The incorporation of phenyllactic acid is dependent on the concentration of magnesium ions, an important parameter in ribosomal systems, as shown in Figure 31. Panel (a) shows the magnesium dependence for phenylalanine incorporation and panel (b) shows the dependence in a similar system for phenyllactic acid incorporation. Both are optimal in the range between 10 and 15 mM Mg^{2+} .

The relevance of the Phelac incorporation to protein synthesis is further indicated by the effect of chloramphenicol and gougerotin. These inhibitors of protein synthesis act by inhibiting the action of peptidyl transferase (Monro and Vazquez, 1967). As shown in Table 6 both of these compounds inhibit the Phelac incorporation. The degree of inhibition is comparable to the degree to which they inhibit polyphenylalanine formation under similar conditions.

FIGURE 31: MAGNESIUM DEPENDENCE OF INCORPORATION
OF PHE AND PHELAC.

The incubation system was as described in Methods, but the concentration of magnesium was varied. Aliquots (0.05 ml) contained either phenylalanyl-tRNA (a) or phenyllactyl-tRNA (b) (equal concentrations as described in Methods) with or without poly U. After 15 min. at 30° C samples were treated with EDTA and RNase, precipitated with TCA, and counted as described in Methods.

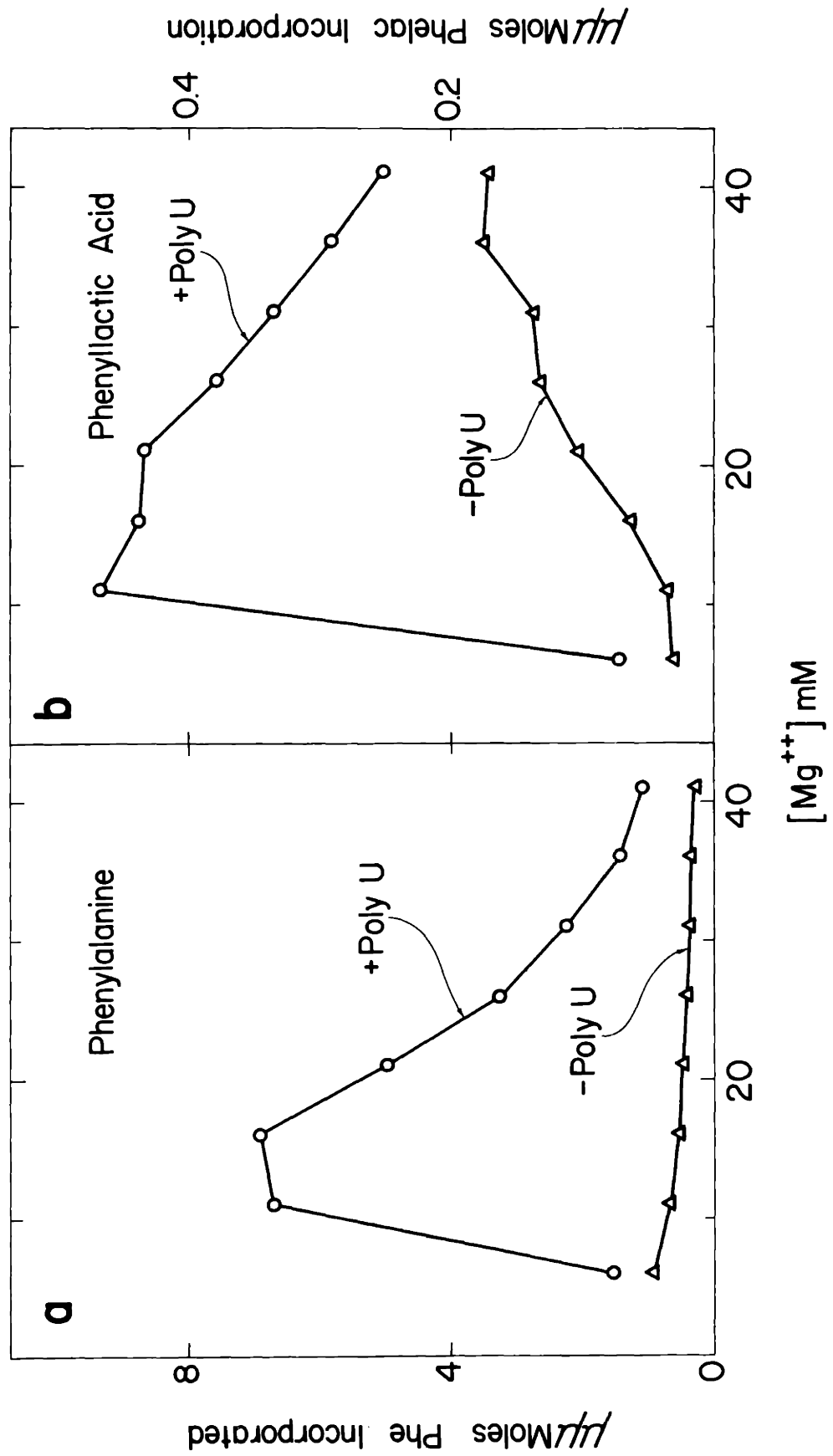


Figure 31

TABLE 6
EFFECT OF PROTEIN SYNTHESIS INHIBITORS ON
THE INCORPORATION OF PHELAC AND PHENYLALANINE

		μ MOLES INCORPORATED	% INHIBITION
		+ Poly U	- Poly U
<hr/>			
A.	Phelac		
	Complete system	0.50	0.07
	+ Chloramphenicol (2 mM)	0.35	0.08
	+ Gougerotin (2 mM)	0.13	0.08
B.	Phenylalanine		
	Complete system	5.10	0.36
	+ Chloramphenicol (2 mM)	2.38	0.24
	+ Gougerotin (2 mM)	1.22	0.32

The complete system was as described in Methods, using in similar mixtures either phenyllactyl-tRNA (A) or phenylalanyl-tRNA (B). Samples (0.05 ml) were incubated with and without poly U for 15 min. at 30° C, then assayed by the RNase-cold TCA procedure described in Methods. The percent inhibition was calculated after subtracting the minus poly U incorporations.

All of these properties indicate that the observed incorporation does not result from some non-specific adsorption of radioactivity. Nor is it due simply to the binding of Phelac-tRNA to the ribosome, protecting it from RNase digestion in the assay. This last possibility is clearly eliminated by (1) the controls on the assay described under Methods, (2) the inability of GMP-PCP to substitute for GTP, even though GMP-PCP can substitute for GTP in ribosome binding of tRNA, and (3) the inhibition by gougerotin. Gougerotin does not inhibit poly U-directed Phe-tRNA binding to ribosomes (Vazquez and Monro, 1967). (4) The incorporation assayed by hot TCA precipitation.

The requirements for the reaction and the action of the inhibitors suggest that the incorporation results from a ribosomal polymerization which involves the action of peptidyl transferase. These data can be interpreted in two alternative ways: (1) Phelac might be incorporated only into N-terminal positions of a polyphenylalanine chain, in the manner reported by Hervé and Chapeville. (2) In a reaction analogous to the reaction of α -hydroxypuromycin, phelac-tRNA might be incorporated into internal positions, producing a polymer containing ester bonds. The following data support the second alternative.

Alkaline Lability

After completion of the incorporation and treatment of the product with EDTA and ribonuclease, it was exposed briefly to 0.67 N NaOH at 0° C before precipitation with a large excess of TCA. As shown in Figure 32 this treatment results in rapid disappearance of the TCA precipitable material. This is a real lability and is not simply due to a change in the ionic conditions under which the TCA precipitation takes place, since there are intermediate points on the curve in Figure 32.

This behavior contrasts with that of polyphenylalanine, the TCA precipitability of which is not affected by much harsher treatment. For example, treatment of the polyphenylalanine product, formed in a similar system, with 1 N NaOH for 2 hours at 40° C resulted in no loss of TCA precipitable radioactivity.

Alkaline digestion of the TCA precipitated Phelac-containing material produces mainly free phenyllactic acid. As shown in Figure 33, the major component in the alkali-treated material coelectrophoreses and cochromatographs on silica gel in two solvent systems with standard phenyllactic acid. The behavior of the

FIGURE 32: ALKALINE TREATMENT OF PHELAC PRODUCT.

Incorporation from phelac-tRNA was carried out as described in Methods in a volume of 0.25 ml. After 45 min. incubation at 30° C, 0.05 ml 0.2 EDTA and 0.05ml 1 mg/ml RNase were added, followed by a second incubation at 30° C for 15 min. Carrier BSA (2 μ l, 30% w/v) was added and the sample divided into 0.05 ml aliquots which were chilled to 0° C. Cold (0° C) 1 N NaOH (0.1 ml) was added to each aliquot, except one, the zero time point. After the appropriate time (abscissa) at 0° C, 2 ml cold 10% TCA containing 0.5% casamino acids was added and the samples millipore filtered and counted as described in Methods.

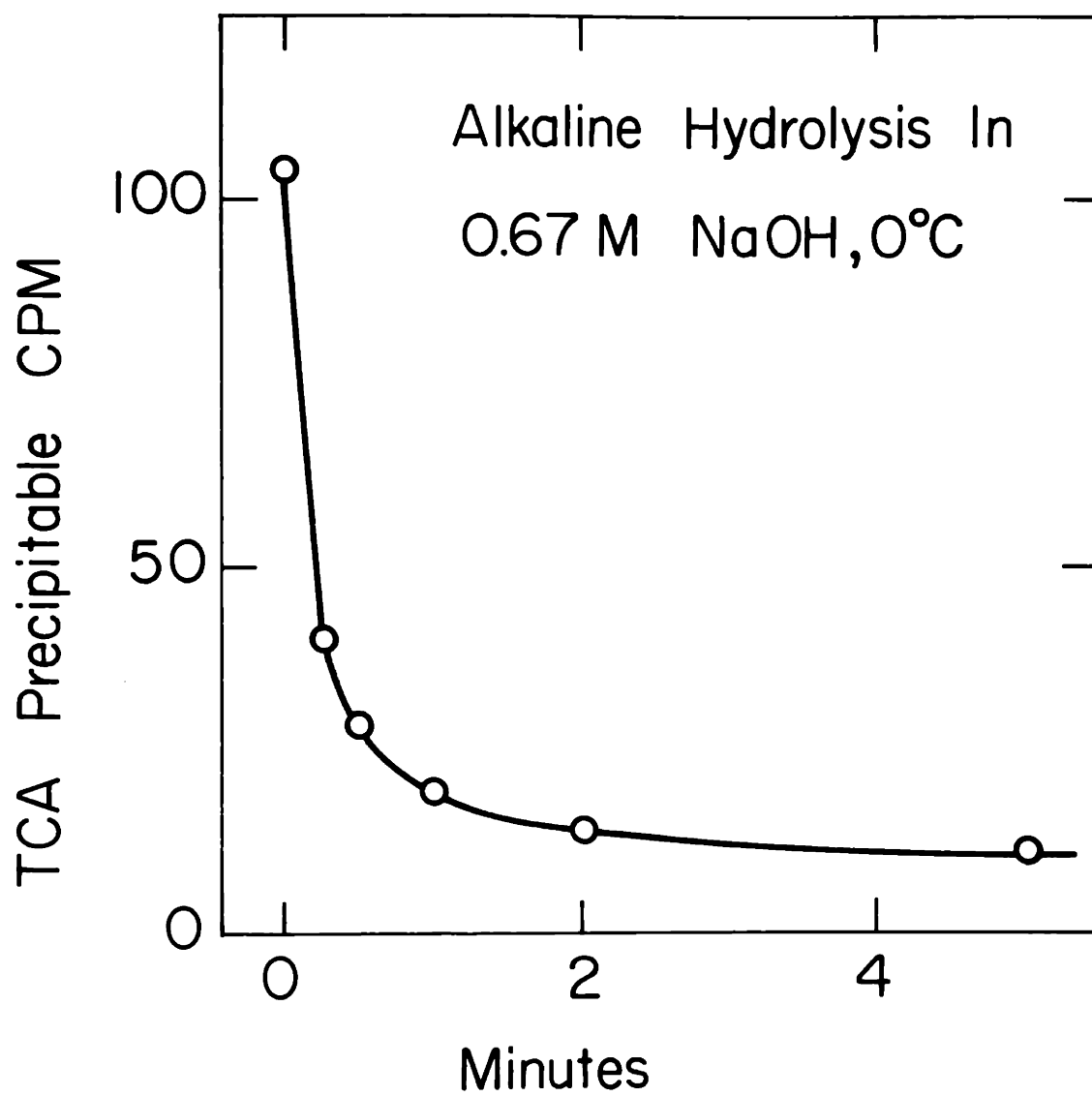


Figure 32

FIGURE 33: CHARACTERIZATION OF THE ALKALINE
DIGESTED PHELAC PRODUCT.

Incorporation from phelac-tRNA was carried out as described in Methods. Aliquots (1 ml) were incubated with and without poly U, then treated in parallel. After 60 min. incubation at 30° C, 0.2 ml 0.2 M EDTA and 0.2 ml 1 mg/ml RNase were added, and samples incubated 15 min. at 30° C. Cold 10% TCA (2 ml) was added and the precipitate collected by centrifugation. The precipitates were washed twice with 5 ml cold 5% TCA by vigorous mixing on a Vortex mixer followed by centrifugation. TCA was removed by lyophilization overnight, and the residue was taken up in 0.1 ml 1 N KOH and incubated 45 min. at 40° C. The samples were neutralized with 1 N HClO₄, and after chilling to 0° C, the precipitated KClO₄ was removed by centrifugation. Carrier phenyllactic acid and phenylalanine (20 µg each) were added. Aliquots of each of these samples were then subjected to chromatography and electrophoresis as follows. Chromatography (ascending) was carried out on Eastman silica gel G thin layers in two different solvent systems. (a) isoamyl alcohol/acetone/acetic acid/water (25:25:1:1). (b) n-butanol/acetic acid/water (78:5:17). Electrophoresis (c) was carried out on Whatman 3 MM paper, in buffer composed of 25 ml pyridine and 10 ml glacial acetic acid per 2.5 liters (pH 5.3), for three hours at 23 V/cm. Silica gel plates were cut into 1 cm strips which were placed in 20 ml toluene based scintillator and counted in a liquid scintillation spectrometer. Electrophoreograms were counted on a Vanguard gas flow strip counter. Dotted lines on chromatograms are controls which were treated in the same way, but

FIGURE 33 (continued)

from which poly U was deleted from the incorporation
system

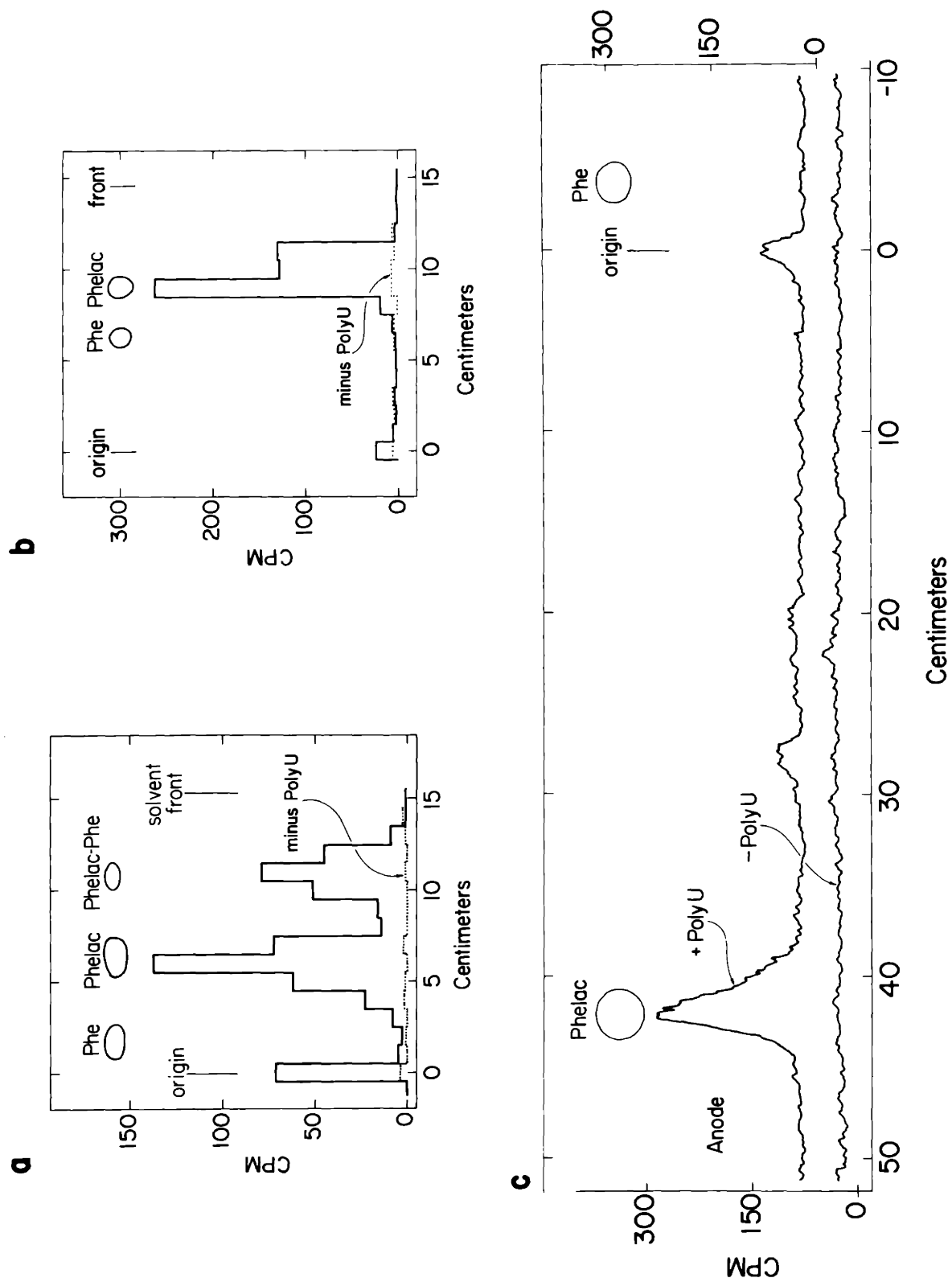


Figure 33

remainder of the material suggests that it may be a heterogeneous mixture of short oligophenylalanine chains containing phenyllactic acid at the N terminus. The important point however is that alkaline treatment does produce mainly free phenyllactic acid.

This behavior is consistent with the proposal that the incorporation results from the formation of a polymer of phenyllactic acid containing ester bonds, but would not be expected of a product in which Phelac is present only at N terminal positions in a polypeptide. In that case Phelac would be joined to the polymer through a peptide bond and would not be removed by the mild alkaline treatment used here.

The observation that free Phelac is released by alkaline treatment eliminates one trivial model, where Phelac might be located only at the N termini of very short (2-3) oligophenylalanine chains which are TCA precipitable only by virtue of the adenosine residue which remains esterified at the carboxyl terminus. Thus alkaline treatment would remove the adenosine and render the oligopeptide acid soluble. However the product of alkaline digestion in that case would be a Phelac-containing oligopeptide, not free Phelac.

Incorporation of the type observed by Hervé and Chapeville can be observed in this system by adding to

the incubation mixture some unlabelled phenylalanyl-tRNA in addition to the labelled Phelac-tRNA. In the experiment described in Table 7 (^{14}C)-labelled phelac-tRNA and unlabelled Phe-tRNA were mixed in various proportions and the products were examined for alkaline lability. The total amount of tRNA present is the same in each incubation. The fraction of the input phelac incorporated is approximately constant but as the ratio of Phe to Phelac increases, the fraction of the incorporated phelac which is resistant to alkali increases. This alkali-resistant incorporation is probably of the type described by Hervé and Chapeville and is clearly distinguishable from the alkali-labile incorporation described above. This observation further confirms the supposition that Phelac at N terminal positions in a polypeptide is not removed by mild alkali treatment.

Number of Phelac Residues per Chain

The interpretation by Hervé and Chapeville of their data as representing the incorporation of phenyllactic acid into N-terminal positions exclusively depends very heavily on the result of a single experiment. They attempted to determine the number of Phelac residues per chain by labelling tRNA with ^{32}P and Phelac with ^{14}C and measuring the ratio $^{14}\text{C}/^{32}\text{P}$ in ribosome bound material

TABLE 7

PHELAC INCORPORATION IN THE PRESENCE OF PHE

$(^{14}\text{C})\text{PHELAC}/$ $(^{12}\text{C})\text{PHE}$	$\mu\text{MOLES PHELAC}$ <u>TCA PRECIPITABLE</u>		PERCENT ALKALI LABILE	PERCENT INPUT PHELAC INCOR- RATED
	STANDARD ASSAY	ALKALI		
1:0	0.56	0.01	98	6.7
1:1	0.25	0.16	35	5.9
1:2	0.19	0.13	33	6.9
1:4	0.104	0.086	17	6.1

Incorporation was carried out in the standard system described in Methods. Each sample contained 3 μl of a mixture of solutions of $(^{12}\text{C})\text{phenylalanyl tRNA}$ ($A_{260} = 200$) and $(^{14}\text{C})\text{phenyllactyl-tRNA}$ ($A_{260} = 200$, 28 μmoles phelac per A_{260} unit) in the proportion shown. Duplicate samples were incubated with and without poly U. After 60 min. incubation at 30° C the samples were treated with EDTA and ribonuclease as described in Methods and carrier BSA (0.05 ml, 2.5 mg/ml) was added. Those listed under "alkali" above were treated with 0.5 ml 1 N NaOH for 15 min. at 40° C. All were precipitated with 2 ml cold 10% TCA containing 0.5% w/v casamino acids, millipore filtered, and counted as described in Methods. The values of incorporation without poly U were subtracted to obtain the values listed.

after incubation with and without GTP. The rationale of this experiment is as follows. The ratio $^{14}\text{C}/^{32}\text{P}$ for ribosome bound material in the absence of GTP is supposed to give the ratio for the population of unpolymerized Phelac-tRNA^{Phe}. Since after polymerization each chain is bound to a single tRNA molecule, the presence of more than one Phelac residue per chain should result in an increase in the ribosome bound $^{14}\text{C}/^{32}\text{P}$ ratio after incubation in the presence of GTP. Thus comparison of the ratio with and without GTP allows calculation of the number of Phelac residues per chain.

The data of Hervé and Chapeville are reproduced in Table 8. There is little difference in the ribosome-bound $^{14}\text{C}/^{32}\text{P}$ ratio for Phelac with or without GTP, in contrast to the large difference for phenylalanine. Hence the conclusion by Hervé and Chapeville that only one Phelac residue is incorporated per chain. This is the central point in their argument that Phelac is incorporated only terminally.

The results of similar experiments are shown in Table 9. In these experiments tRNA was labelled with ^3H and Phelac with ^{14}C . Ribosome bound material was isolated by millipore filtration in Experiment I and by sedimentation through sucrose in Experiment II. In both experiments the presence of GTP results in a pronounced increase in the $^{14}\text{C}/^3\text{H}$ ratio in the ribosome bound fraction. In Experiment I this increase is relative to the value

TABLE 8
 DETERMINATION OF AVERAGE NUMBER OF RESIDUES
 PER CHAIN

(Reproduced from Hervé, G. and F. Chapeville
J. Mol. Biol. 13,757 (1965))

	$^{14}\text{C}/^{32}\text{P}$ Ratio in the Ribosomal-Bound Material		Average Number of Residues per Chain
	- GTP	+ GTP	
Phe	0.360	3.30	9.5
Phelac	0.425	0.415	1.0

For experimental details see original publication.

TABLE 9

DETERMINATION OF AVERAGE NUMBER OF PHELAC RESIDUES

PER CHAIN

	RIBOSOME BOUND DPM ¹⁴ C		RIBOSOME BOUND DPM ³ H		+ POLY U ¹⁴ C/ ³ H
	+ Poly U	- Poly U	+ Poly U	- Poly U	
EXP. I					
Complete System	1693	382	17146	19511	0.099
Minus GTP, PEP	428	180	22468	23928	0.021
plus Goug-erotin (1 mM)	1000	456	21535	19940	0.046
E X P. II					
Complete System	908	75	20745	15877	0.044
Minus GTP, PEP					
plus GMP-PCP (0.5 mM)	136	80	16460	18571	0.0083
plus Goug-erotin (1 mM)	480	105	19838	21963	0.024

The complete incubation mixture (0.1 ml) in both experiments contained 0.11 M Tris-HCl, pH 7.4; 0.011 M magnesium acetate; 0.096 M NH_4Cl ; 0.5 mM GTP; 4 mM PEP; 0.0089 mg S-100 protein; 0.13 A_{260} units ribosomes; and 2.4 μg poly U; and (^{14}C) phelac-(^3H)-tRNA (37 μmoles phelac, 38200 dpm ^{14}C , 5.20×10^6 dpm ^3H). Duplicate samples were incubated with and without Poly U for 45 min. at 30° C.

Experiment I: Samples were diluted with 2 ml cold buffer

TABLE 9 (continued)

(0.1 M Tris-HCl, pH 7.4, 0.011 M magnesium acetate, 0.09 M NH_4Cl) and passed through a millipore filter. The filter was then washed three times with 3 ml cold buffer, dried, dissolved in 10 ml Brays, and counted in a liquid scintillation spectrometer, using the channels ratio quench correction method described in Methods.

Experiment II. Samples were diluted with 4 ml cold buffer as in Experiment I and layered onto 8 ml 10% sucrose in the same buffer. Tubes were centrifuged for 6 hours at 41,000 rpm in a Spinco SW 41 rotor at 2° C. Supernatant was removed to within 0.5 ml of the bottom of the tube by repeated aspiration and washing of the upper portions of the tube. The ribosomal pellet was resuspended in the bottom 0.5 ml of supernatant by shaking, dissolved in 10 ml Brays, and counted as in Experiment I.

obtained by merely deleting GTP and PEP. In Experiment II the comparison is between values obtained with GTP and with GMP-PCP, the GTP analog which promotes enzymatic binding of tRNA to ribosomes but will not support polymerization. The indicated number of residues per chain is 4.7 in Experiment I and 5.3 in Experiment II.

The presence of 1 mM gougerotin partially prevents this increase in $^{14}\text{C}/^3\text{H}$ ratio with GTP addition. This indicates there are fewer residues of Phelac per chain in the presence of gougerotin than in its absence. Since gougerotin inhibits polypeptide synthesis by inhibiting peptidyl transferase, this observation is not consistent with incorporation of Phelac only at terminal positions, but further indicates the presence of several Phelac residues per chain.

For comparison with the data of Hervé and Chapeville the ratios were calculated in the same manner as theirs. However straightforward interpretation of these ratios depends on the assumption that the ratio obtained without GTP represents the ratio for the population of Phelac-tRNA^{Phe}. The data of Table 9 indicate that this is not the case. Under all conditions the amount of (^3H)tRNA bound is approximately the same whether poly U is present or not. Thus most of the tRNA binding observed is non-specific. Furthermore the $^{14}\text{C}/^3\text{H}$ ratio for the input Phelac tRNA preparation is 0.0075. According to the

assumption on which the experiment is based this ratio should be considerably enriched in ^{14}C in the material bound to ribosomes (by a factor of 20 if Phelac tRNA^{Phe} is 1/20 of the total population of tRNA's). The enrichment in Experiment I was a factor of 3, and the enrichment in Experiment II was a factor of 1.1. Clearly the ratio measured in the absence of GTP does not represent the ratio for the Phelac-tRNA^{Phe} population.

The data of Hervé and Chapeville suffer from the same defect. This is not clear in the data provided in Table 8 because neither the input $^{14}\text{C}/^{32}\text{P}$ ratio nor the data obtained without poly U are given. However in a similar experiment with phenylalanine in another publication Haenni and Chapeville (1966) give the input ratio for Phe charged tRNA as 0.22 and the ratio for material bound to ribosomes without GTP as 0.360 (same as in Table 5). The ^{14}C is enriched by a factor of 1.6, again indicating substantial non-specific binding of tRNA.

Therefore in these experiments the ratio of interest does not represent the ratio for specifically bound aminoacyl- or peptidyl-tRNA. Rather the ^3H dpm can be considered simply a measure of the number of ribosomes present, assuming the non-specific tRNA binding is not affected by the absence of GTP or the presence of ouabain. Then the observed ratio differences reflect differences in the number of Phelac residues bound per ribosome, not per

nascent chain. Thus in order to interpret the data as reflecting the number of residues per chain one further assumption is required: that for each Phelac tRNA bound in the absence of GTP one polymer chain is formed when GTP is added. [This assumption is acknowledged by Haenni and Chapeville (1966).]

This assumption certainly weakens the argument based on this data. However whether or not the conclusions which have been drawn from this data are justified, it is clear that the results shown in Table 9 are quite different from those of Hervé and Chapeville. Since this experiment is central to their argument, the difference can be taken as justification for the contention that the alkali lability of the incorporated Phelac reflects incorporation of Phelac into internal positions, not simply the N-terminal incorporation reported by those authors.

Two possible explanations might be proposed to account for the difference of these results from those of Hervé and Chapeville. The conditions under which those authors converted Phe-tRNA to Phelac-tRNA were harsher (more acidic) than the conditions used here. It is possible that their conditions cause some damage to the tRNA which prevents it from functioning as an acceptor but allows it to function as an initiator, for which the structural requirements may be less stringent. Certainly

damage to the RNA can occur. Hervé and Chapeville reported that after nitrous acid treatment tRNA could be charged with Phe only to the extent of 20 to 30% of the charging of untreated tRNA. In one attempt to reproduce the preparation of Hervé and Chapeville, Phelac-tRNA was obtained which was not functional in this system and was unable to bind to ribosomes in the presence of poly U. However the deamination conditions used here are considerably milder. The data of Carbon (1965) indicate that after 30 min. treatment under these milder conditions on the average less than 0.2 nucleotides per tRNA molecule are deaminated. Treatment of tRNA^{Phe} in this way decreases its amino acid acceptance by 30% (see Methods).

Secondly, the difference may be due to the presence of larger amounts of phenylalanine in the supernatant preparation of Hervé and Chapeville. The data of Table 7 indicate that the presence of phenylalanine does allow the incorporation of Phelac in an alkali-stable form which may be N-terminal.

The Phage R17 RNA-Directed System

The incorporation of phenyllactic acid into an alkali-labile polymer probably results from the action of phenyllactyl-tRNA as a peptidyl acceptor during ribosomal polymerization, resulting in incorporation of Phelac into internal positions in a polymer. This is analogous to the action of the α -hydroxy analogs of puromycin and further supports the conclusion that an α -hydroxyacyl acceptor can be used by peptidyl transferase acting in a manner analogous to its action during protein synthesis.

In this section the previous observations are extended to an *in vitro* protein synthesizing system in which a natural mRNA, the RNA of bacteriophage R17, is accurately translated. The single stranded RNA of this bacteriophage acts as mRNA *in vivo* for the synthesis of three proteins: (1) the coat protein, (2) the maturation protein, a minor component of the virus particle and (3) the phage RNA polymerase (see Gussin *et al*, 1966). In *in vitro* systems this RNA also directs the synthesis of these three proteins, but the major product is the coat protein (Lodish and Robertson, 1969; Capecchi, 1966; Nathans, 1965).

An amber mutant of phage R17 (amB₂) contains a chain terminating codon (UAG) at the seventh position of the coat protein cistron (Tooze and Weber, 1967). In *in vitro* systems the RNA of this virus directs the synthesis of the N-terminal hexapeptide fragment of the coat protein, which is released from the ribosome (Zinder *et al*, 1966; Capecchi, 1967b). The amino acid sequence of this fragment is fMet-Ala-Ser-Asn-Phe-Thr.

If an α -hydroxyacyl-tRNA is substituted for the corresponding aminoacyl-tRNA in the *in vitro* system the resulting coat protein fragment contains an ester bond at a specific position. This results from the incorporation of an α -hydroxy acid (phenyllactic acid or lactic acid) into an internal position in the polypeptide chain in place of the corresponding amino acid (phenylalanine or alanine, respectively).

This observation constitutes an unambiguous demonstration that α -hydroxy acids can be incorporated into internal positions and that peptidyl transferase of a single ribosome can use both α -hydroxyacyl and α -aminoacyl acceptors. This synthesis takes place in an *in vitro* system under the direction of a natural messenger RNA and under ionic conditions (7 mM magnesium) which are known to allow accurate initiation, translation, and termination, and which are therefore probably similar to the actual *in vivo* conditions.

Synthesis of the Coat Hexapeptide

The outline of the experiment is shown in Figure 34. The incorporation system, described in detail in Methods, contained R17 amB₂ RNA as message, a preparation of ribosomes composed of about 60% native subunits from which initiation factors had not been removed, and an RNA-free S-100. The concentration of magnesium ions in the incubation mixture was 7 mM. One mixture contained (³⁵S)-fMet-tRNA and the other five (non-radioactive) aminoacyl-tRNAs (alanine, serine, asparagine, phenylalanine, and threonine). In similar mixtures one of the aminoacyl tRNAs was replaced by its α-hydroxyacyl analog, formed by nitrous acid deamination of the aminoacyl-tRNA. In one case lactyl-tRNA^{Ala} was substituted for alanyl-tRNA, and in the other case phenyllactyl-tRNA^{Phe} was substituted for phenylalanyl-tRNA.

The hexapeptide product of each of these incubations was isolated by the method of Capecchi (1967) as described in Methods, then further purified by electrophoresis. The fMet labelled, acid soluble, hydrophobic, N-blocked peptide products formed in the three incorporations are shown in Figure 35. The major product formed in the presence of amino acids only and isolated in this way is the hexapeptide (Zinder *et al*, 1966; Capecchi, 1967) (Panel (a) in Figure 35). This peak does not appear in similar systems

FIGURE 34: OUTLINE OF THE EXPERIMENT DESCRIBED
IN THE TEXT.

The heavy circles represent (^{35}S)fMet, the
only radioactive amino acid. The solid circles
represent α -hydroxy acids.

Ester Incorporation into RI7 Coat Peptide

Hexapeptide: fMet-Ala-Ser-Asn-Phe-Thr

RI7 amB₂ RNA, Ribosomes, S-100, GTP,
³⁵S fMet-tRNA, 7 mM Mg⁺⁺

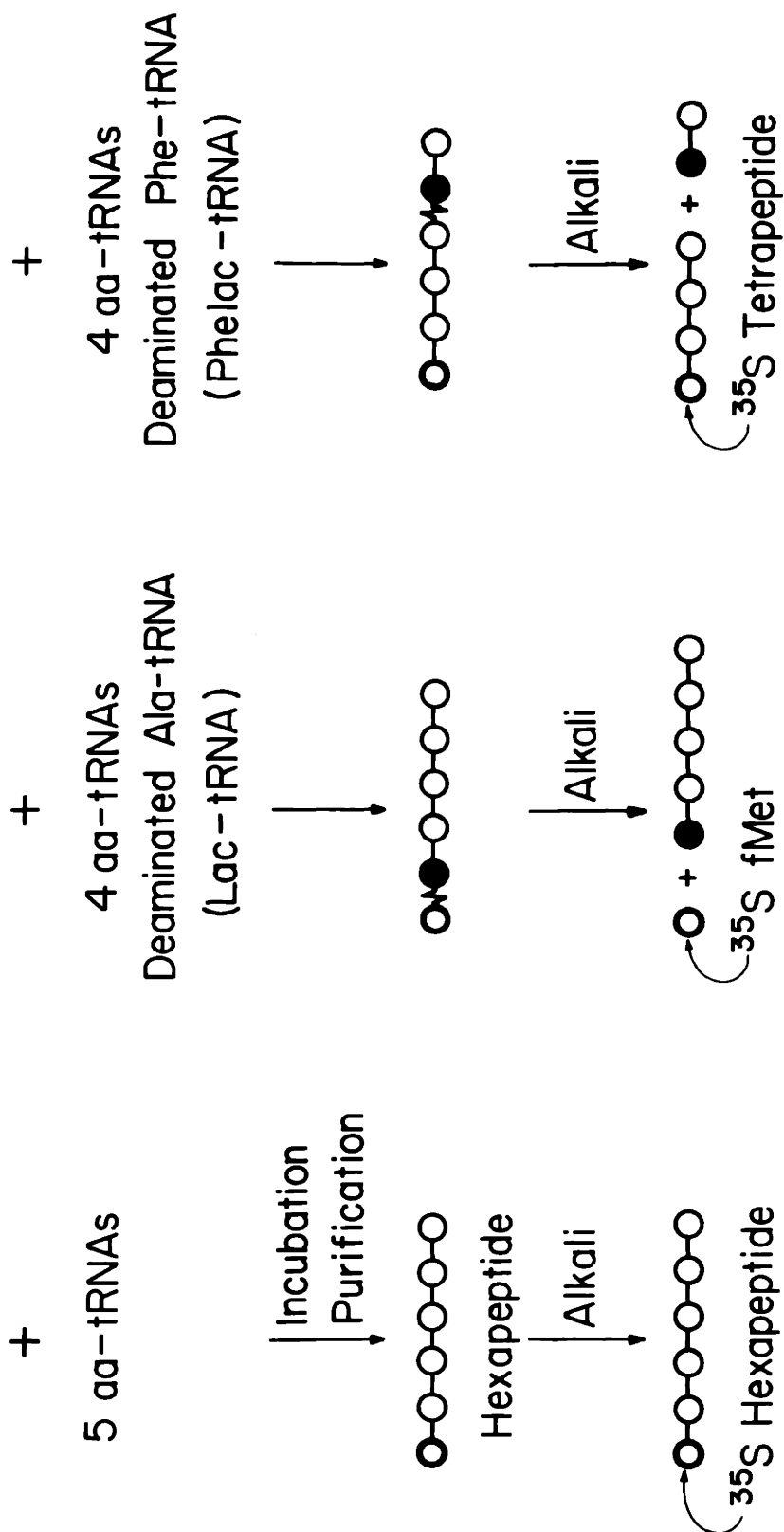


Figure 34

FIGURE 35: PURIFICATION OF THE HEXAPEPTIDE PRODUCTS.

After 30 min. of incubation, the TCA soluble, N-blocked peptides were isolated by the method of Capecchi (1967). The hexapeptide was further purified by electrophoresis on paper (Whatmann 3MM) in a buffer composed of 14 ml pyridine and 12.5 ml glacial acetic acid per liter (pH 4.8), at 28 v/cm for 5 hours. The hexapeptide region was eluted.

- (a) Products formed from amino acids only.
- (b) Lac-tRNA^{Ala} substituted for Ala-tRNA.
- (c) Phelac-tRNA^{Phe} substituted for Phe-tRNA.

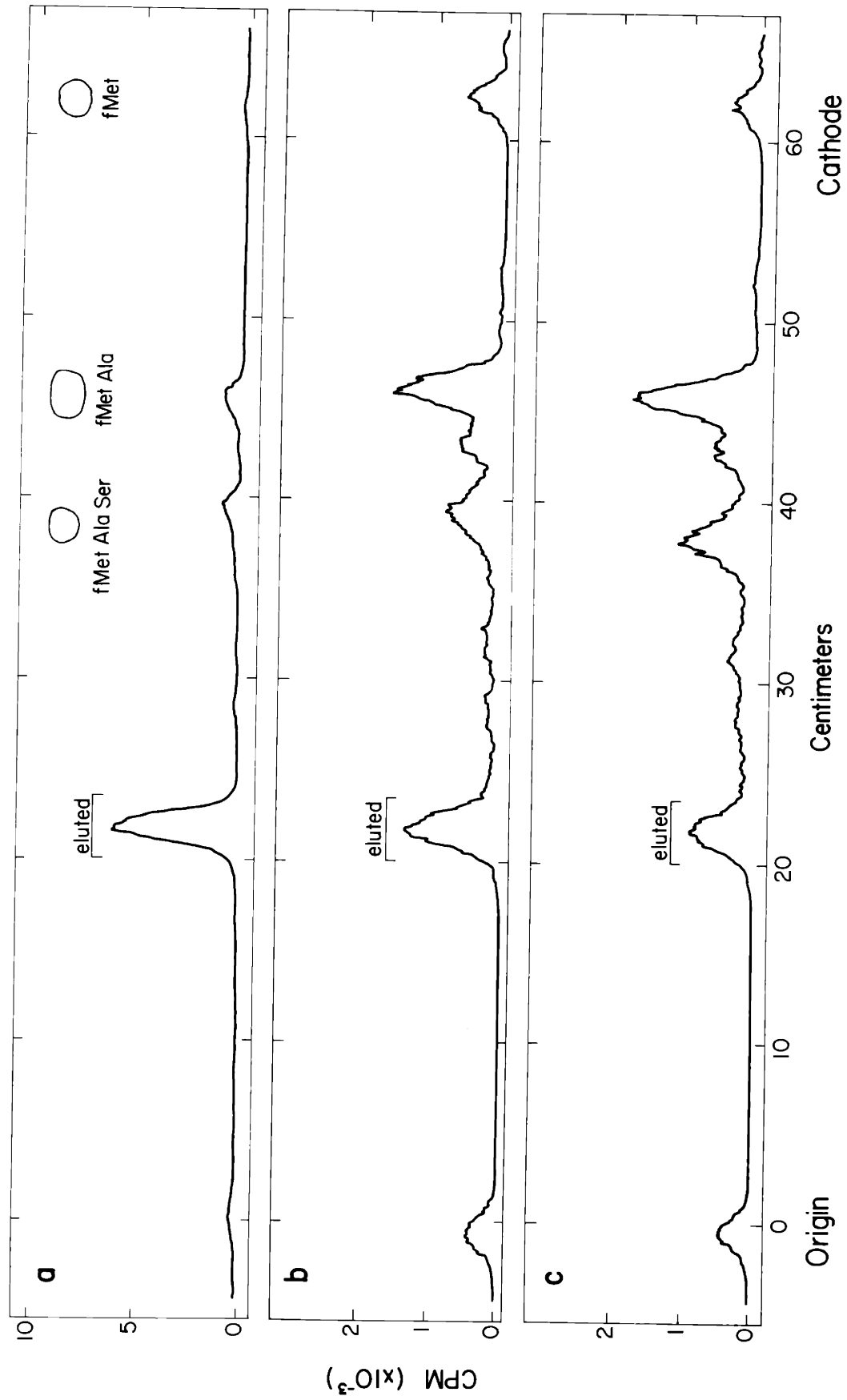


Figure 35

in which the RNA of wild type R17 is substituted for that of the amber mutant. A radioactive product with the same mobility was also formed in each of the incubation mixtures which contained α -hydroxyacyl-tRNAs (Panels (b) and (c) in Figure 35). In addition, there are smaller peptide products which may be formed by proteolytic digestion of the released hexapeptides. The material in the hexapeptide region was eluted from each electrophoreogram and tested for the presence of ester bonds by treatment with 1 M triethylamine, pH 12.5.

The triethylamine-treated and untreated hexapeptide products were analyzed by electrophoresis. The autoradiogram shown in Figure 36 contains the products of the reaction together with mobility standards. Under conditions of this electrophoresis N-blocked peptides containing one to six amino acids are resolved with a mobility which varies inversely with the molecular weight of the peptide (Kuechler and Rich, 1970). In Figure 36 the origin is marked by radioactive uridine (U). The numbers indicate the number of amino acids in the various peptides, with dotted circles indicating the positions of the non-radioactive standards.

As shown in Figure 36, the hexapeptide synthesized in the presence of all five amino acids plus labelled fMet was unaffected by the alkaline treatment. However,

FIGURE 36: CHARACTERIZATION OF THE HEXAPEPTIDE PRODUCTS.

The hexapeptides purified as described in Figure 35 were tested for the presence of ester bonds by treatment with 1 M triethylamine, (pH 12.5) at 35° C for 20 min. Triethylamine treated and untreated samples were then subjected to electrophoresis at 4° C on a cellulose thin layer (E. Merck) in the same pyridine-acetate buffer, pH 4.8, for 105 min. at 30 v/cm. Radioactivity was located by autoradiography. Nonradioactive standards were located with the platonic iodide reagent of Toennies and Kolb (1951).

Standards: (1) fMet, (2) fMet Ala, (3) fMet Ala Ser, (U) uridine (^{14}C). Uridine marks the origin, corrected for endosmotic buffer flow. (4) Tetrapeptide, (6) hexapeptide.

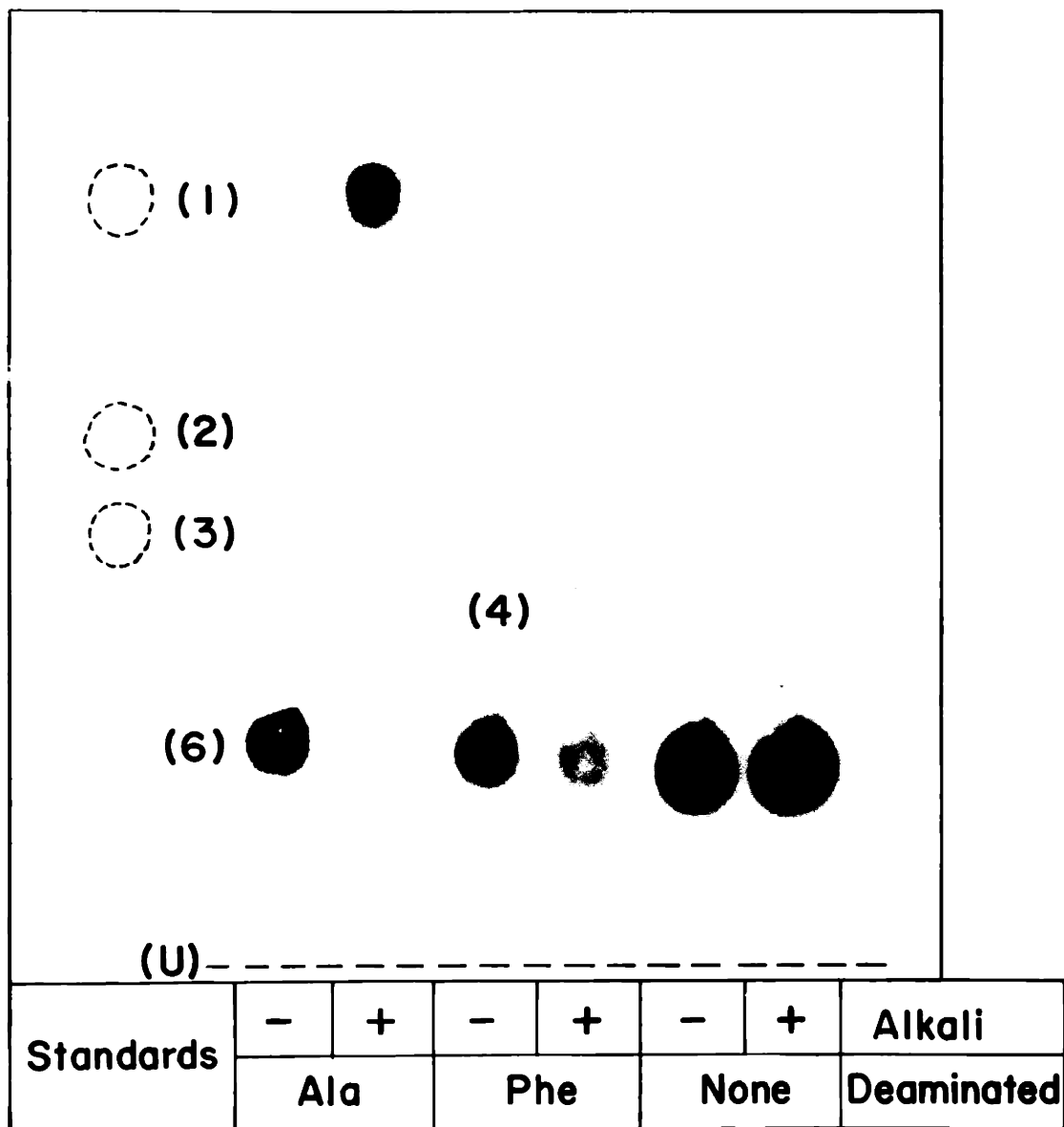


Figure 36

the hexapeptide made in the presence of deaminated Ala-tRNA contained an alkali labile ester bond between the first and second positions and was hydrolyzed to labelled fMet. Similarly, the hexapeptide made in the presence of deaminated Phe-tRNA contained an ester bond between the fourth and fifth positions and was hydrolyzed to the labelled tetrapeptide, fMet-Ala-Ser-Asn. This is most simply interpreted as indicating the incorporation of lactic acid at the second position in place of alanine, and phenyllactic acid at the fifth position in place of phenylalanine.

The characterization of the product of alkaline hydrolysis of the phenyllactic acid hexapeptide as the tetrapeptide is based on the fact that its mobility is that expected for the tetrapeptide by interpolation of the known mobilities of the standards fMet, fMet Ala, fMet Ala Ser, and the hexapeptide. These mobilities are indicated in Figure 37. The closed circles represent the mobilities of the synthetic standards, and the open circle the hexapeptide made in the presence of all six amino acids. The mobilities represented by the crosses are those of spots obtained by partial pronase digestion of the hexapeptide and they are placed at the molecular weights corresponding to their presumed structures. The arrow indicates the mobility of the product of alkaline

FIGURE 37: RELATIVE ELECTROPHORETIC MOBILITY
OF PEPTIDES AS A FUNCTION OF MOLEC-
ULAR WEIGHT.

Electrophoresis was carried out as described in Figure 37. The mobilities of fMet and the synthetic marker peptides (fMet Ala and fMet Ala Ser) are indicated by filled circles. The hexapeptide synthesized in the *in vitro* system directed by R17 amB₂ RNA in the presence of all required amino acids is indicated by an open circle. The crosses indicate the mobilities of spots obtained by partial pronase digestion (0.3 mg/ml pronase, pH 8, 37° C for 30 min.) of the (³⁵S) fMet-labelled hexapeptide. The arrow indicates the mobility of the material formed by alkaline hydrolysis of the hexapeptide made in the presence of Phelac-tRNA.

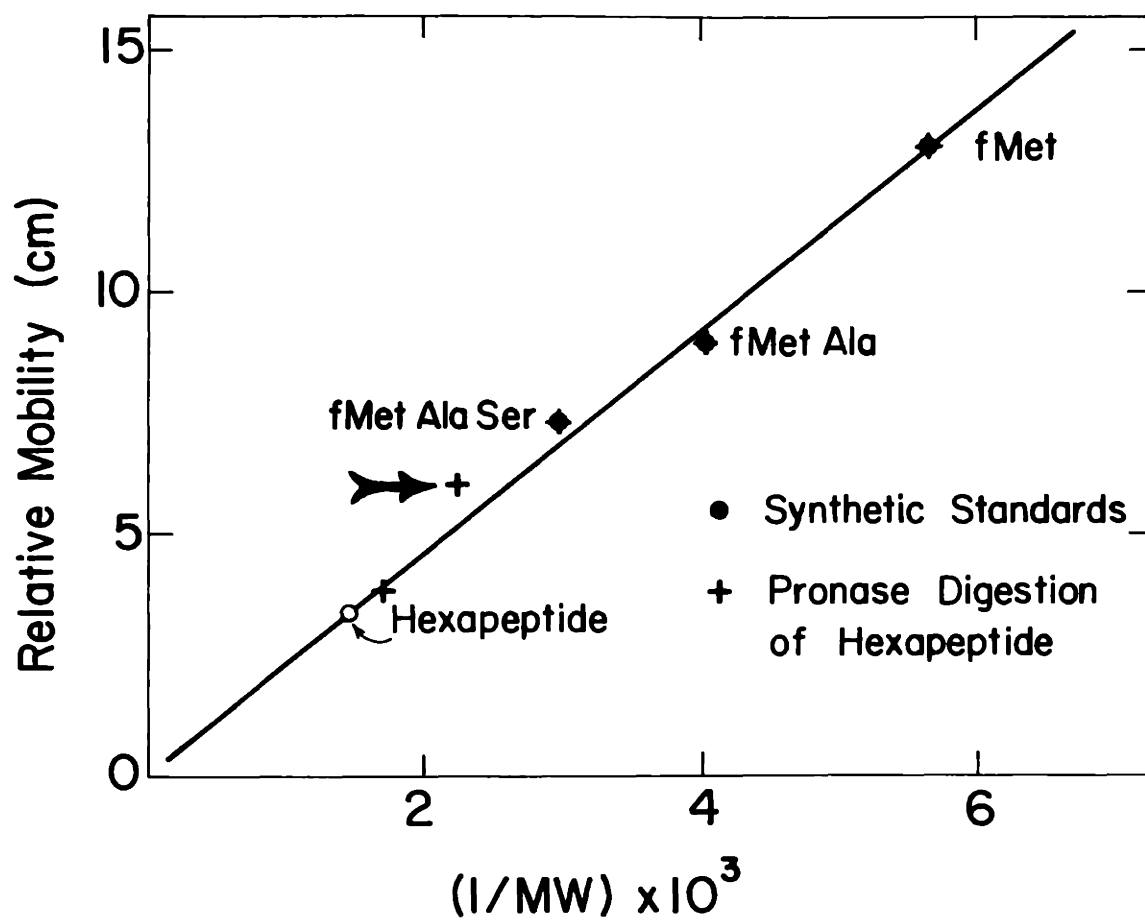


Figure 37

hydrolysis of the Phelac hexapeptide. It is approximately the same as that of one of the products of pronase digestion of the hexapeptide, and both are in the range of mobility expected of the tetrapeptide.

The radioactivity in all of these spots remains in formylmethionine. Extended pronase digestion of the hexapeptide made in the presence of phenyllactic acid gave complete conversion of the labelled material to fMet, as was the case for the hexapeptide made with all amino acids.

It is apparent in Figure 35 that the yield of hexapeptide in the presence of an α -hydroxyacyl-tRNA is lower than the yield with only aminoacyl-tRNAs. The yield of alkali-labile hexapeptide in the presence of Lac-tRNA is lower by a factor of 5, and the yield in the presence of Phelac-tRNA is lower by a factor of 12, than the yield of hexapeptide from aminoacyl-tRNAs. This reduction might be explained in part by a preference of peptidyl transferase itself for the α -amino acid over the α -hydroxy acid. This was indicated in the previous section by comparison of ψ hydroxypuromycin with puromycin. It was found that the K_m for ψ hydroxypuromycin is higher than that for puromycin by a factor of 5 to 6, and the maximal reaction velocity is lower by a factor of 9. The reduced yield of hexapeptide may also be due

in part to the lower efficiency of T-factor complex formation with α -hydroxyacyl-tRNAs, reported below.

It is also apparent in Figure 36 that the hexapeptides made in the presence of the α -hydroxyacyl-tRNAs are partially resistant to the alkaline treatment. In the case of the alanine substitution 70-80% of the hexapeptide was alkali-labile, while 40-50% of the phenylalanine-substituted material was alkali labile. The alkali resistant hexapeptide made in these two incubations is probably due to the presence of small amounts of alanine and phenylalanine in the system. Even though the S-100 was freed of amino acids it is possible that peptidases in the system brought about the release of these amino acids which were subsequently activated to aminoacyl-tRNA and incorporated.

T-Factor Recognition of Phelac-tRNA

The above observations demonstrate that the ribosomal peptidyl transferase can use either α -hydroxyacyl- or aminoacyl-tRNAs during polypeptide synthesis. This suggests the possibility that this flexibility could be made use of *in vivo* to make polypeptides containing ester bonds. Whether this can occur or not depends on (1) whether α -hydroxyacyl-tRNAs exist or can be formed in the cell, and (2) whether they can gain access to the ribosome.

It is presently believed that aminoacyl-tRNA binds to ribosomes in the form of a ternary complex with one of the supernatant factors (T_u) and GTP (Ravel, 1967; Lucas-Lenard and Haenni, 1968; Haenni and Lucas-Lenard, 1968; Ertel *et al*, 1968; Erbe *et al*, 1969). This complex cannot be formed with deacylated tRNA. Furthermore it has been reported that the analogous complex in a wheat embryo system cannot be formed with nitrous acid treated Phe-tRNA (Jerez *et al*, 1969). If this were also true in *E. coli*, it would presumably prevent α -hydroxyacyl-tRNAs from functioning by excluding them from the ribosome. In the *in vitro* systems described here it is conceivable that the T factor requirement is less stringent than it may be *in vivo*. Therefore the ability of phenyllactyl-tRNA to form the T-factor complex was examined directly. These experiments were performed with Dr. H. Weissbach.

The preparation of Phelac-tRNA used in these experiments contained, in addition to Phelac, some other species which presumably arise by rearrangement of the intermediate carbonium ion during nitrous acid deamination (see Figure 23). Though these products were not identified, one of them is presumably α -phenyl- β -hydroxypropionic acid (Roberts and Regan, 1953). The other is probably cinnamic acid (H. Neumann, Personal Communication). These

rearrangement products must be considered less likely to interact with T-factor than is phenyllactic acid, but their presence should be kept in mind when considering the following results.

Formation of the ternary complex can be assayed by a millipore filtration technique. Formation of the ternary complex is preceded by formation of a ($T_u \cdot \text{GTP}$) complex. This can be formed from ($T_u \cdot \text{GDP}$) either by incubation with factor T_s plus GTP, or by incubation with phosphoenol pyruvate and pyruvate kinase (Weissbach *et al*, 1970). The complex ($T_u \cdot \text{GTP}$) is retained by millipore filters (Allende and Weissbach, 1967). However the ternary complex ($T_u \cdot \text{GTP} \cdot \text{aatRNA}$) is not retained (Gordon, 1968). Thus the formation of the ternary complex can be assayed using radioactive GTP by determining the decrease in millipore-bound radioactivity on adding tRNA to the ($T_u \cdot \text{GTP}$) complex.

The effect of Phelac-tRNA on the millipore binding of ($T_u \cdot \text{GTP}$) is shown in Table 10. For this experiment purified tRNA^{Phe} was used to prepare phenylalanyl-tRNA, which was then treated with nitrous acid. ($T_u \cdot [^3\text{H}] \cdot \text{GTP}$) was formed by incubating ($T_u \cdot \text{GDP}$) with phosphoenol pyruvate and PEP kinase. Its binding to millipore filters was then examined after incubation with and without Phelac-tRNA. Phelac-tRNA causes a decrease in the amount of millipore bound ($T_u \cdot \text{GTP}$). Phelac-tRNA, which has been deacylated by alkaline treatment, has no

TABLE 10
INTERACTION BETWEEN [T_v -GTP] AND Phelac-tRNA

<u>tRNA</u>	<u>μMOLES GTP BOUND TO FILTER</u>	<u>μ MOLES TERNARY COMPLEX</u>
None	25	--
Phe-tRNA (28 μ moles)	14	11
Phelac-tRNA (37 μ moles)	18	7
Phelac-tRNA, KOH treated (37 μ moles)	24	1

The experimental procedure is described under Methods. The second incubation contained Phe-tRNA or Phelac-tRNA as listed. The "KOH treated" sample was incubated for 10 min. at 37° C in 0.01 M KOH, then neutralized with HCl before assay.

such effect. Thus Phelac-tRNA is able to interact with the ($T_u \cdot GTP$) complex. The preparation of Phelac-tRNA contained less than 0.1% phenylalanyl-tRNA, so the interaction is clearly not due to contaminating Phe-tRNA.

In order to determine whether the interaction indicated in Table 10 reflects the formation of a productive ternary complex, the effect of T_u plus T_s on the ribosome binding of Phelac-tRNA was examined. It can be seen in Table 11 that the ribosome binding of (^{14}C) Phelac-tRNA (determined by binding to a millipore filter) is stimulated more than two fold by T_u plus T_s . This effect is somewhat less than the effect on Phe-tRNA binding, but the two effects are of comparable magnitude.

Thus it can be concluded that under the conditions of these assays (10 mM magnesium) nitrous acid-treated Phe-tRNA is able to form a functional ternary complex with T_u and GTP. It is not clear from these data whether the complex is formed with Phelac-tRNA or with one of the rearrangement products described above. It is likely that Phelac is involved. In any case the T factor experiments support the interpretation that the incorporation of Phelac in the R17 RNA system involves the intermediate formation of a T-factor complex. The efficiency of complex formation is lower than obtained with Phe-tRNA, and this may account in part for the decreased yield in the presence of α -hydroxyacyl-tRNA.

TABLE 11
RIBOSOME BINDING OF Phelac:
EFFECT OF T_U AND T_S

A. <u>Phelac-tRNA</u>		<u>$\mu\mu$MOLES BOUND</u>
Complete System, 2 min.		1.5
- T_U , T_S	"	0.8
-Ribosomes	"	0.2
-Poly U	"	0.2
B. <u>Phe tRNA</u>		
Complete System, 2 min.		3.3
Complete System, 1 min.		2.3
- T_U , T_S	"	0.7
-Ribosomes	"	0.3

The complete system and millipore filter assay for binding to ribosomes are described under Methods. Incubation was for the time period shown, at 23° C. In A, incubation mixtures contained 7.0 $\mu\mu$ moles of (^{14}C) Phelac-tRNA^{Phe}. In B, incubation mixtures contained 7.4 $\mu\mu$ moles of (^{14}C)Phe-tRNA^{Phe}.

It is possible, therefore, that such a complex could be formed, probably with low efficiency, *in vivo*.

DISCUSSION

The activity of α -hydroxy analogs of puromycin indicates that *E. coli* ribosomes have the capacity to catalyze the formation of ester bonds when provided with an α -hydroxyacyl acceptor. A variety of characteristics of that reaction, which resemble the characteristics of the puromycin reaction, lead to the conclusion that it is catalyzed by the same enzyme which catalyzes the puromycin reaction, namely peptidyl transferase. However, those results left open the question of whether or not the ribosome is able to introduce ester bonds in a system in which messenger RNA is directing the synthesis of polypeptide chains under physiological conditions.

The ability of α -hydroxyacyl-tRNAs to function as acceptors in message RNA directed ribosomal polymerizations confirms and extends those conclusions. This ability is demonstrated by the results of this section in two systems. Phenyllactic acid is incorporated from phelac-tRNA into an alkali labile polymer synthesized by ribosomes under the direction of poly U. The data support the interpretation that Phelac is incorporated into internal positions in a polyester. The incorporation of α -hydroxy acids into internal positions in a polypeptide is shown unambiguously by the *in vitro* synthesis of a phage R17 coat protein peptide containing ester bonds at specific positions.

Thus peptidyl transferase can catalyze the formation of ester bonds when acting in a manner exactly analogous to its action during protein synthesis. Unlike the reactions of puromycin and its analogs these reactions take place in combination with all of the auxiliary ribosomal substrate binding functions. The α -hydroxyacyl acceptor substrates are esterified to tRNAs and are bound to ribosomes through codon specific interactions. In addition the functions of chain initiation, translocation, and chain termination are made use of in the R17 RNA system, indicating that the formation of the ester bond takes place in the midst of a normal series of ribosomal manipulations with no irreversible gross distortion of the system.

The incorporation of lactic acid and phenyllactic acid into the R17 coat polypeptide further demonstrates that peptidyl transferase of a single ribosome can catalyze both ester and peptide bond formation. This point could not be proven unambiguously for the reactions of the puromycin analogs. Though unlikely because of the broad range of similarities of the two reactions, it was possible that the puromycin and α -hydroxypuromycin reactions were catalyzed by two different subsets of the ribosome preparation. However, in the R17 RNA system a polymer is synthesized which contains both peptide and ester bonds in the same chain. Since a polypeptide chain is

believed to be assembled on a single ribosome and not released from the ribosome until a terminator codon is encountered (Gilbert, 1963; Bretscher, 1968), it is clear that a single ribosome can use both α -hydroxyacyl and α -aminoacyl acceptors interchangeably.

It might also be proposed that the reaction of α -hydroxy-puromycin is catalyzed, not by peptidyl transferase, but by a hypothetical chain terminating enzyme. The process of chain termination involves the hydrolysis of the ester bond between the polypeptide chain and tRNA, that is, transfer of the polypeptide chain to a hydroxyl acceptor, namely water. It is conceivable then that a special chain terminating enzyme could use as an acceptor a hydroxyl group attached to an acyl adenosine residue, i.e., α -hydroxy-puromycin. The similarity of the peptidyl transferase and "terminase" reactions in terms of ionic requirements and inhibitor sensitivities might be explained by the proximity of the respective active centers on the ribosome and sensitivity of both centers to conditions in a common region around them which might include inhibitor binding sites. However, this proposal is unlikely in view of the results of this section. In this case the α -hydroxyacyl acceptors are substituted for aminoacyl acceptors in the midst of a series of events (ribosome binding, translocation, etc.) which normally occur during polypeptide chain elongation.

It is unlikely that a hypothetical chain terminating enzyme is invoked in place of peptidyl transferase to add an α -hydroxyacyl residue onto a chain which is then further elongated by the normal process.

In fact it is likely that no special chain terminating enzyme exists, but that the hydrolysis is catalyzed by peptidyl transferase itself. (Vogel, et al, 1969; Tompkins, et al, 1970; Capecchi and Klein, 1969). In that case the usual requirement of the enzyme for an adenosine moiety in the acceptor substrate might be relieved in some way by the presence of a chain termination factor. This conclusion is based mainly on the fact that inhibitors of peptidyl transferase also affect the termination reaction. However, this argument is subject to the mild reservation cited above, i.e., that two separate active sites in close proximity may be involved. If the chain termination reaction is catalyzed by peptidyl transferase then it is necessary that the enzyme have the ability to transfer the polypeptide chain to either an amino group or a hydroxyl. This flexibility is demonstrated by the observations described here. Including the observation of Scolnick, et al, (1970), there now exists a continuous spectrum of peptidyl transferase acceptor substrates: α -aminoacyl-tRNA, α -hydroxyacyl-tRNA, puromycin, α -hydroxypuromycin, ethanol, and water. The existence of this continuous spectrum enhances the likelihood that the first and last members of the series

as listed are substrates for the same enzyme.

The results of this section demonstrate that α -hydroxyacyl-tRNAs are incorporated *in vitro* during the accurate translation of a natural messenger RNA (R17 RNA). These experiments were carried out under ionic conditions which are known to allow accurate initiation, translation, and termination. It is especially important that the concentration of magnesium ions is approximately the same as that which is optimal (7.5 mM) for the *in vitro* synthesis of enzymatically active lysozyme in the system of Salser, et al, (1967). These conditions may therefore be similar to the actual *in vivo* environment. Furthermore, Phelac-tRNA participates normally, probably even to the extent that it is able to form a productive T-factor complex [phelac-tRNA-T_U-GTP]. Accordingly, these results suggest that if an α -hydroxyacyl-tRNA can be formed *in vivo*, it could be incorporated into a polypeptide chain, forming a protein containing labile ester bonds.

DISCUSSION

Experiments have been described which indicate that *E. coli* ribosomal peptidyl transferase is able to use as acceptor substrates molecules containing either an amino group or a hydroxyl group, and consequently to catalyze the formation of both amide and ester bonds. Since this flexibility can be demonstrated in an *in vitro* system in which a natural mRNA is translated under conditions which are probably compatible with the *in vivo* environment, the ability of the ribosome to make ester linkages could conceivably be made use of *in vivo*. Whether it is or not, the flexibility of the enzyme is of interest because of its relevance to consideration of its mechanism of action.

Synthesis of Esters *In Vivo*

In one sense this flexibility is probably utilized *in vivo*. As discussed above, it is likely that peptidyl transferase catalyzes the hydrolysis of peptidyl-tRNA ester bond during the process of chain termination. This reaction constitutes the transfer of the polypeptide chain to a hydroxyl acceptor.

It is also possible that proteins containing α -hydroxyacyl residues could be synthesized on ribosomes. The experiments described in this dissertation indicate this can occur provided only that an α -hydroxyacyl-tRNA exists or can be formed in the cell. No such α -hydroxyacyl-tRNA has ever been isolated from *E. coli*, but this should

not be taken as strong evidence that none exists, since it could be present in very small amounts. In fact it has been shown by Yegian, et al, (1966) that a large fraction (25 to 50%) of some species of tRNA molecules in *E. coli* is esterified to something other than amino acids. Some of these contain short nascent polypeptide chains and some may contain N-acylated amino acids (a portion, but not all, is formylmethionine). However, a sufficient portion of the esterified material is unidentified to allow for the possibility of the presence of some α -hydroxy acids.

By analogy with the formation of N-formylthionyl-tRNA it is likely that such a species would be formed by modification of an aminoacyl-tRNA. The specificity of the normal aminoacyl-tRNA synthetases probably prevents the direct activation of α -hydroxy acids. For example, phenyllactic acid cannot be activated by a crude *E. coli* supernatant, nor does it interfere with the activation of phenylalanine (Hervé and Chapeville, 1965).

The incorporation of α -hydroxy acids would result in the formation of proteins containing alkali labile ester linkages. It is interesting to note that fragments smaller than the protomer are liberated by treating β -galactosidase at pH 12 (Steers, et al, 1965). These fragments are not separate units in the native enzyme, but are covalently joined in a single polypeptide (Ulmann, et al, 1968). It is therefore apparent that β -galactosidase contains an

unusually labile linkage. This may possibly be an ester, or, perhaps more likely, a peptide bond which is strained by the tertiary structure of the protein.

The Catalytic Mechanism of Peptidyl Transferase

It may be useful to consider the relevance of the observations presented in this dissertation to the mechanism of action of peptidyl transferase. The ability of the enzyme to catalyze the formation of both amide and ester bonds parallels the similar flexibility of most proteolytic enzymes, which can hydrolyze both amide and ester bonds (Dixon and Webb, 1964). This similarity may be more than trivially superficial.

In addition to the hydrolysis reactions, proteolytic enzymes can catalyze acyl transfer reactions which are entirely analogous to the ribosomal peptidyl transferase reaction. A striking example of this is the formation of di- and tripeptides of methionine from methionine-isopropyl-ester, catalyzed by chymotrypsin (Brenner, et al, 1950). This reaction is formally analogous to the ribosomal formation of polypeptides from transfer RNA esters of amino acids.

The catalytic mechanism of chymotrypsin is understood in some detail. The tertiary structure of the enzyme, and of complexes of the enzyme with substrate analogs, has been determined by X-ray crystallography (Blow, et al, 1969;

Steitz, et al, 1969). Based on the geometry of the active site and the extensive background of information which is available from more conventional enzymology, Blow, et al, (1969) proposed a detailed catalytic mechanism. The most important features of this mechanism are the involvement of the hydroxyl group of a serine residue (at position 195) and the imidazole group of a histidine residue (at position 57). During the hydrolysis of a peptide bond the carboxyl group is transferred to serine 195 where it is esterified until transferred to an acceptor, generally water. The imidazole nitrogen of histidine 57 acts as a general acid and general base catalyst respectively in the two transfer steps.

Once an acyl residue is esterified to serine 195 it can be transferred to a variety of acceptors in addition to water. The accepting group can be either an amino group or a hydroxyl. If the acceptor is an amino acid the result is the formation of a peptide bond, as in the reaction of the methionine ester described above. In these acyl transfer reactions the hydroxyl of serine 195 can be considered analogous to the 3'- (or 2'-) hydroxyl of the terminal adenosine of peptidyl-tRNA, and the overall analogy to the ribosomal peptidyl transferase reaction is apparent.

Inward and Jencks (1965) examined the reactivity of

a series of amines and alcohols as acceptors of the acyl residue from furoylchymotrypsin (i.e., the serine 195 ester of furoic acid). Comparing a series of simple alkyl amines and the corresponding alcohols they found that the rate constant for transfer to the amine is higher than the rate constant for transfer to the alcohol by a factor of about 3 to 5. The magnitude and direction of this difference are comparable to the difference between puromycin and the hydroxyl analogs in reactions catalyzed by peptidyl transferase. The V_{\max} for puromycin is higher than that for the hydroxyl analogs by a factor of 7 to 9.

The ability of peptidyl transferase to transfer an acyl residue to either an amino group or a hydroxyl group is consistent with the operation of a general base type catalysis similar to that which is operative in the acyl transfer reactions catalyzed by chymotrypsin. A further similarity of the two enzymes is to be found in their pH-dependence. The activity of peptidyl transferase increases with pH between pH 7 and pH 8.5. The pH-dependence for the acyl transfer reactions of chymotrypsin is similar, but shifted slightly to lower pH (Inward and Jencks, 1965). For chymotrypsin the involvement of a group of pK 7.0 is indicated. The group which is responsible for this pH-dependence is almost certainly the imidazole of histidine 57,

the group which acts as a general base during catalysis. The similar pH-dependence of peptidyl transferase is again consistent with the operation of such a general base type mechanism.

None of these similarities between chymotrypsin and peptidyl transferase can be taken as definitive evidence that a similar catalytic mechanism is operative. Nevertheless the similarities are quite suggestive. The attractiveness of the idea that such a catalytic mechanism is used by peptidyl transferase is enhanced by the apparent ubiquity of the mechanism among various proteases. Active sites with essentially identical geometry to that of chymotrypsin have been observed in the structures of elastase (Shotton and Watson, 1970) and subtilisin (Wright, et al, 1969). Chymotrypsin and elastase are from mammalian sources (cow and pig, respectively) but subtilisin is isolated from a bacterium, *Bacillus amyloliquefaciens*. The presence of essentially identical active sites in enzymes produced by organisms as evolutionarily unrelated as mammals and bacteria is a striking example of convergent evolution. In view of the implied desirability of this kind of catalytic mechanism and its ability to do the job required of peptidyl transferase, it is reasonable to propose as a working hypothesis that a similar mechanism is utilized by peptidyl transferase.

If an imidazole residue (or N-terminal α -amino group) is involved as a general base in the catalysis it might be possible to identify it by affinity labelling technique similar to that which was used to identify histidine 57 in chymotrypsin (Ong, et al, 1964). A great deal is known about the substrate specificity of peptidyl transferase, so it should be possible to design substrate analogs (for example analogs of puromycin) containing reactive groups which might become covalently bound to the hypothetical imidazole residue or to other groups which are present in the active site. The chloroketone used by Ong, et al, (1964) may be a useful model; others are provided by the diazonium compounds which react with histidine, tyrosine, and tryptophane residues in the active site of Staphylococcal nuclease (Cuatrecasas, 1970).

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