Ribosome-Catalyzed Ester Formation*

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ABSTRACT: Analogs of puromycin in which the α-amino group is replaced by a hydroxyl group were synthesized by condensation of puromycin aminonucleoside with L-(−)-3-phenyllactic acid and by nitrous acid deamination of puromycin. *E. coli* ribosomal peptidyl transferase is able to transfer *N*-formylmethionine from fMet-tRNA or the terminal hexanucleotide fragment of fMet-tRNA to the α-hydroxyl groups of these 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 the ribosomes at high temperatures destroys the ability to catalyze both reactions to the same degree.

The product of the analog reaction is an ester, as shown by its lability under mildly alkaline conditions. It is otherwise indistinguishable from fMet-puromycin. Peptidyl transferase can thus catalyze the formation of an ester bond as well as the usual peptide bond. The significance of this observation with respect to the mechanism of action of peptidyl transferase and its possible involvement in polypeptide chain termination are discussed.

Peptide bond formation in protein synthesis is catalyzed by an enzyme, peptidyl transferase, which appears to be an integral part of the large ribosomal subunit (Monro, 1967; Maden *et al.*, 1968). In addition to the normal reaction involving transfer of the nascent polypeptide chain from peptidyl-transfer RNA to the α-amino group of aminoacyl-tRNA, this enzyme also catalyzes peptidyl transfer reactions in which the acceptor is the antibiotic puromycin, which resembles the aminoacyl end of tRNA (Yarmolinski and de la Haba, 1959). Because of the similarity of the puromycin reaction to the normal reaction it has proved useful in examining the properties of peptidyl transferase as distinguished from the specific substrate binding functions of the ribosome. In the course of studying the structural requirements for this reaction (Neumann *et al.*, 1968), we have made derivatives of puromycin in which the α-amino group is replaced by a hydroxyl group. Study of the activity of this modified puromycin derivative in an *Escherichia coli in vitro* system has led to the discovery that the ribosome can catalyze the attachment of an amino acid to this α-hydroxyl group through an ester linkage almost as efficiently as it catalyzes the formation of a peptide bond with the α-amino group. It is of interest that most of the proteolytic enzymes which catalyze the reverse reaction, the hydrolysis of peptide bonds, are also esterases (Dixon and Webb, 1964). This may have great bearing upon our attempts to understand the mechanism of action of the ribosomal peptidyl transferase. In the present report we present some of the evidence for ester formation and make a comparison of its efficiency relative to peptide bond formation.

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Materials and Methods

Puromycin was obtained from Cyclo Chemical Co. and Nutritional Biochemicals. Chloramphenicol was a gift from Parke Davis and Co. Gougerotin was generously supplied as a gift by Dr. T. Kanzaki of Takeda Chemical Industries, Ltd.

Puromycin Analogs. α-Hydroxypuromycin was obtained by nitrous acid deamination of puromycin. Puromycin dihydrochloride (100 mg) was dissolved in 20 ml of cold (0°) 1 N HCl. To this was added, slowly with stirring, 20 ml of cold 1 N $NaNO_2$. The mixture was incubated overnight at 0°. NaOH (4 ml, 5 N) was then added, and the mixture extracted with three 50-ml aliquots of ethyl acetate. Combined ethyl acetate layers were washed with 25 ml of H_2O and dried at room temperature under reduced pressure. 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.

Demethoxy-α-hydroxypuromycin was synthesized from puromycin aminonucleoside (Sigma Chemical Co.) and L-(−)-3-phenyllactic acid (Aldrich Chemical Co.). This synthesis will be described in detail elsewhere, as will the syntheses of *N*-acetylpuromycin, α-chloropuromycin, and α-methoxypuromycin.

Incubation System. [^{35}S]Methionine of specific activity 1000 to 5000 mCi per mmole was prepared according to the method of Sanger *et al.* (1964). It was used to synthesize [^{35}S]fMet-tRNA from stripped *E. coli* K12 soluble RNA (General Biochemicals) by the method of Hershey and Thach (1967).

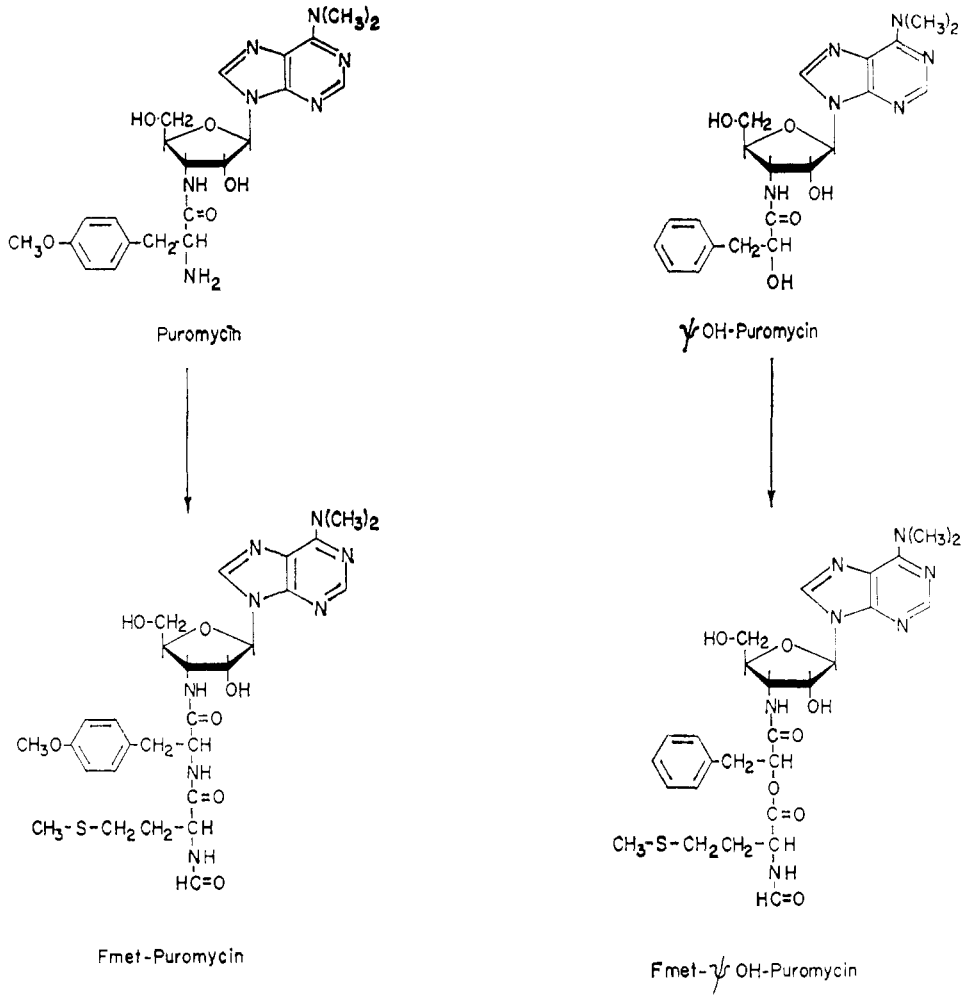

FIGURE 1: Formulas of the products of the reaction of formylmethionyl-tRNA with either puromycin or demethoxy-α-hydroxypuromycin.

[^{35}S]fMet T_1 oligonucleotide fragment was prepared from fMet-tRNA as described by Marcker (1965).

Ribosomes washed with 2 M NH_4Cl and crude (unfractionated) initiation factors were prepared from *E. coli* Q 13 as described by Anderson *et al.* (1967). Postribosomal supernatant (S-100) was prepared according to Hershey and Thach (1967). Bacteriophage R17 RNA was a gift from P. Lu.

fMet Fragment Reaction (*Monro and Marcker, 1967*). Prior to methanol addition, the reaction mixture (0.1 ml) contained 0.06 M Tris-HCl buffer (pH 8.1, measured at 0°), 0.4 M KCl, 0.02 M magnesium acetate, 0.006 M β-mercaptoethanol, 13.8 A_{260} units/ml of ribosomes, and the formylmethionyl T_1 fragment (50,000 dpm/ml, or as noted in figure legends).

The reaction was initiated with an equal volume of methanol (control) or a methanolic solution of puromycin, hydroxypuromycin, or ψ-hydroxypuromycin. Concentrations of puromycin, hydroxypuromycin, and ψ-hydroxypuromycin were determined spectrophotometrically in 0.1 N HCl solution (λ_{max} 267.5 nm; ε 2.0 × 10^4 for all three compounds). Incubation was at 0° for time periods noted in figure legends. The reaction was terminated with 25 μl of 0.1 M $BeCl_2$, and 0.1 ml of 0.3 M sodium acetate (pH 5.5) saturated with $MgSO_4$, and then 1.5 ml of ethyl acetate were added. The mixture was shaken at room temperature for 15 sec and centrifuged briefly. The ethyl acetate layer (1 ml) 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 A_{260} units/ml of [^{35}S]fMet-tRNA, containing 2.3 × 10^5 dpm/A_{260} unit and about 60% formylated; 41 A_{260} units/ml of ribosomes and 0.8 mg/ml of crude initiation factors; 7.2 A_{260} units/ml of bacteriophage R 17 RNA; and 1.0 × 10^{-3} M ψ-hydroxypuromycin. Aliquots (0.1 ml) were incubated for 15 min at 30°. The reaction was terminated with 25 μl of 0.1 M $BeCl_2$. Sodium acetate (0.1 ml, 0.3 M, pH 5.5), saturated with $MgSO_4$, and ethyl acetate (1.5 ml) 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. Brinkmann cellulose thin-layer plates were used for electrophoresis at 17 V/cm in sodium formate buffers, pH 3.0, 3.6, 3.9, or 4.2, as noted, all at an ionic strength of 0.2. Radioactivity was located by autoradiography on Kodak Royal Blue X-Ray film. Standard *N*-formylmethionine was located with the platinic iodide reagent described by Toennies and Kolb (1951).

TABLE I: 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[a]	350 dpm	827 dpm
Minus Mg^{2+}	28	16
Minus K^+	28	−3
Minus ribosomes	33	15
Minus ribosomes plus postribosomal supernatant (15 μg of protein)	20	−37
Plus 1 mM chloramphenicol	−39	−15
Plus 1 mM gougerotin	45	5

[a] The complete system was as described under Methods (fMet fragment reaction). Incubation was for 15 min. Blanks, incubated without puromycin or ψ-hydropuroxymycin, have been subtracted.

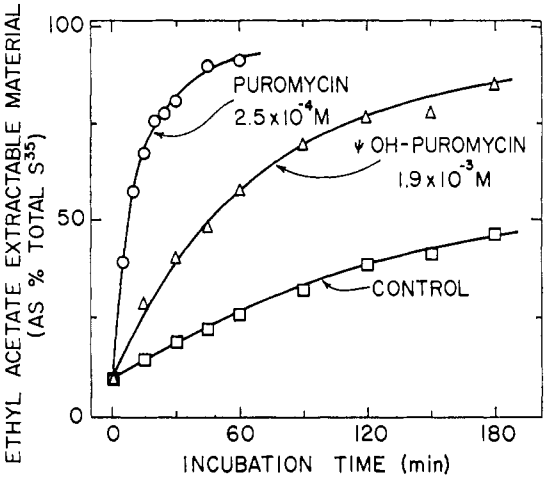

FIGURE 2: Time course of the formylmethionyl T_1 oligonucleotide fragment reaction with ψ-hydroxypuromycin and puromycin. Reaction conditions are described under Methods. The final concentration of puromycin was 2.5×10^{-4} M, and the concentration of ψ-hydroxypuromycin was 1.9×10^{-3} M.

Results

Two closely related analogs of puromycin have been synthesized. One of these, which we call demethoxy-α-hydroxypuromycin (ψ-hydroxypuromycin) differs from puromycin by replacement of the α-amino group by a hydroxyl group and by the lack of the *p*-methoxy group. This compound was prepared by condensation of L-(−)-3-phenyllactic acid to puromycin aminonucleoside in the presence of dicyclohexylcarbodiimide. Its structure is as shown in Figure 1. Proof of its structure is based on evidence from nuclear magnetic resonance, ultraviolet, and infrared spectroscopy of the crystalline synthetic product. The synthesis and characterization of this compound will be described in detail elsewhere.

A compound which behaves similarly to ψ-hydroxypuromycin in the *in vitro* systems described below was prepared from puromycin by nitrous acid deamination. This procedure yields three chromatographically separable products, only one of which is active. We call this compound α-hydroxypuromycin. Roberts and Regan (1953) have shown in similar cases that some rearrangement is to be expected in this reaction. The inactive products are probably formed by such rearrangement and by racemization at the α carbon. They inhibit the activity of the active component.

Demethoxy-α-hydroxypuromycin (ψ-hydroxypuromycin) and α-hydroxypuromycin give similar results in all of the *in vitro* experiments described below. Because of this similarity it is likely that the structure of hydroxypuromycin is the same as that of ψ-hydroxypuromycin except for the presence of the *p*-methoxy group. This group does not appreciably affect the activity of puromycin (Nathans and Neidle, 1963).

Reactions of Puromycin and Its Analogs. The simplest of the puromycin reactions are those involving formation of *N*-formylmethionylpuromycin (fMet-puromycin) from *N*-formylmethionyl-tRNA (Bretscher and Marcker, 1966; Zamir *et al.*, 1966) or an *N*-formylmethionyl hexanucleotide fragment derived from fMet-tRNA by digestion with RNase T_1 (Monro and Marcker, 1967). We have used both of these systems to study the activity of the two puromycin analogs.

We first describe the reactions in the simplest system using the formylmethionyl-tRNA fragment. fMet-puromycin can be assayed by extraction with ethyl acetate from a pH 5.5 aqueous phase (Leder and Bursztyn, 1966; Maden and Monro, 1968). This extraction is specific for uncharged compounds of labeled methionine in which both the amino and carboxyl groups are blocked. When ψ-hydroxypuromycin is incubated with ribosomes and a [^{35}S]formylmethionine labeled oligonucleotide fragment under conditions where fMet-puromycin can be formed, an ethyl acetate extractable product is formed. The kinetics of the reaction are shown in Figure 2 for both puromycin and ψ-hydroxypuromycin. Ethyl acetate extractable material formed in the absence of puromycin or ψ-hydroxypuromycin ("control" in Figure 2) is largely formylmethionine methyl ester as demonstrated by chromatographic comparison with chemically synthesized fMet-methyl ester. The methyl ester formation is independent of ribosomes but is strongly dependent upon pH and the concentration of methanol. The reactions are terminated by $BeCl_2$ which is slightly acidic, and lowers the pH sufficiently to stop the nonenzymatic methanolysis as well as the enzymatic reactions. As shown in Figure 2, almost all of the labeled formylmethionine is converted into ethyl acetate extractable material by puromycin and ψ-hydroxypuromycin. However, ψ-hydroxypuromycin is used in higher concentration. As shown in Table I the stimulation due to ψ-hydroxypuromycin is dependent upon ribosomes, and is not observed when postribosomal 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 (Monro and Vazquez, 1967). In these respects the reaction is identical with the puromycin reaction.

The dependence of the rate of this reaction upon the concentration of ψ-hydroxypuromycin is shown in the Line-

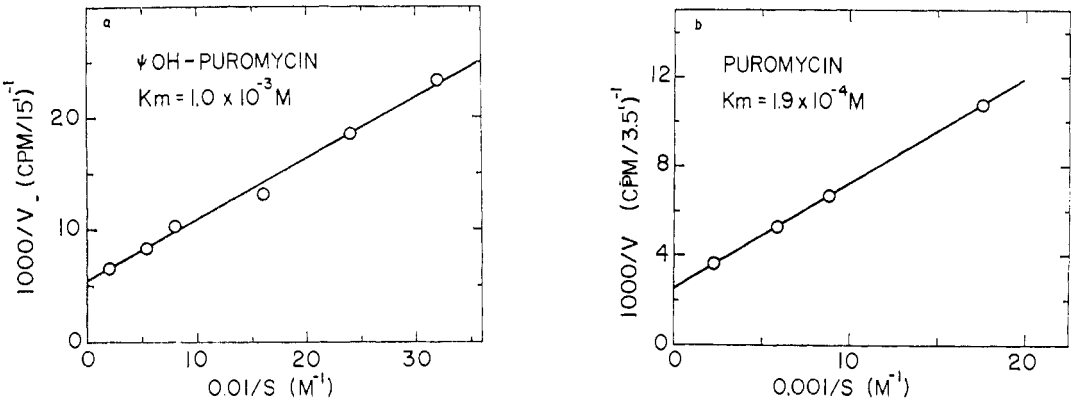

FIGURE 3: Dependence of initial reaction rate upon concentration of ψ-hydroxypuromycin and puromycin. Reaction conditions in the fMet fragment system were as described under Methods. Aliquots (0.2 ml) containing puromycin were incubated for 3.5 min, those containing ψ-hydroxypuromycin were incubated 15 min. A control was also incubated for each of these times without puromycin or ψ-hydroxypuromycin. Each aliquot contained 2670 cpm of formylmethionyl T_1 fragment. The reaction was terminated and assayed as described under Methods. The value obtained for the appropriate control 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 counts per minute in the ethyl acetate aliquot minus control; the units of the abscissa are reciprocal molar concentration of puromycin or ψ-hydroxypuromycin: (a) data for ψ-hydroxypuromycin; (b) data for puromycin.

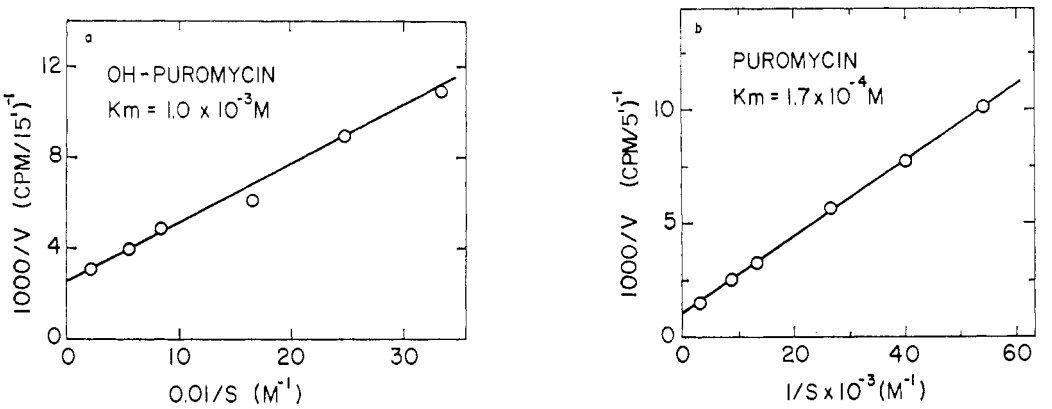

FIGURE 4: Dependence of initial reaction rate upon concentration of hydroxypuromycin and puromycin. Reaction conditions and treatment of data were as described in Figure 3. Aliquots containing puromycin were incubated for 5 min and those containing hydroxypuromycin for either 7.5 min (three highest concentrations) 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: (a) data for hydroxypuromycin; (b) data for puromycin.

weaver–Burk plots of Figure 3. The apparent K_m for ψ-hydroxypuromycin is 1.0×10^{-3} M. In a similar experiment shown in Figure 4 the K_m for hydroxypuromycin was found also to be 1.0×10^{-3} M. The K_m for puromycin under the same conditions is 1.7 to 1.9×10^{-4} M. The ratios of the reaction velocity, V_{max}, for ψ-hydroxypuromycin and hydroxypuromycin to that for puromycin are, respectively, 0.11 and 0.14. The value of the observed K_m for puromycin is independent of the concentration of the T_1 oligonucleotide in the range used in these experiments, but the V_{max} is strongly dependent on oligonucleotide concentration. ψ-Hydroxypuromycin is synthesized from the L isomer of β-phenyllactic acid. However, if racemic DL-β-phenyllactic acid is used, the resulting compound has a K_m twice that of the L isomer (2.3×10^{-3} M) while the V_{max} is roughly the same as that of the L isomer. Thus, the reaction appears to be specific for the L isomer of ψ-hydroxypuromycin, just as the puromycin reaction is specific.

The formation of fMet-ψ-hydroxypuromycin has also been demonstrated in a system more closely resembling *in vivo* conditions, using intact fMet-tRNA bound to ribosomes with bacteriophage R17 RNA in the presence of initiation factors and GTP. As shown in Table II the reaction is dependent upon ribosomes and is also inhibited by chloramphenicol and gougerotin. Methanol is not present in this system.

Characterization of the Product. Figure 5 shows the electrophoretic behavior of the products synthesized in the T_1 fragment reaction. The presence of ψ-hydroxypuromycin results in the appearance of a new component which has a mobility which is very similar to that of fMet-puromcyin at all of the pH values examined. In the range between pH 3.0 and 4.2 any 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 5 the mobility of fMet-puromycin increases between pH 4.2 and pH 3.0

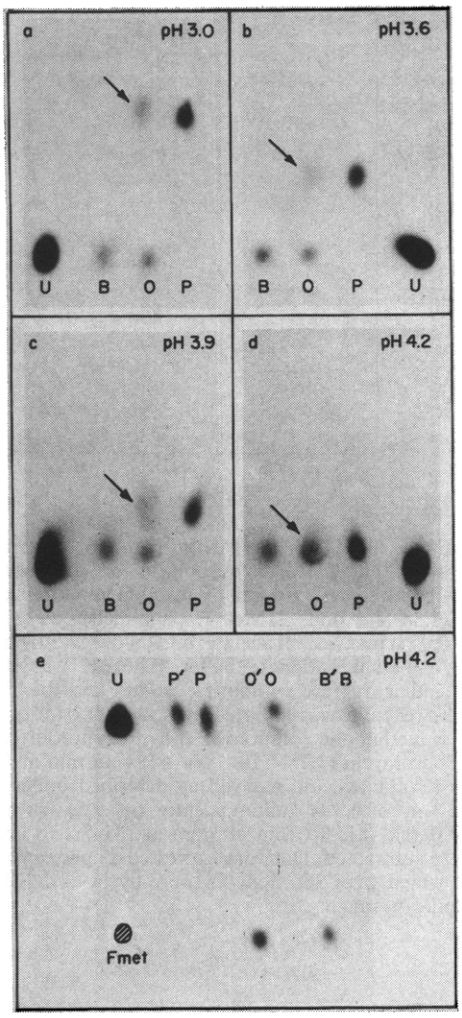

FIGURE 5: Electrophoretic comparison of fMet-puromycin and fMet-ψ-hydroxypuromycin. Products were formed from [^{35}S]fMet-T_1 oligonucleotide under conditions as described under Methods, except that the ribosome concentration was 23 A_{260} units/ml and the T_1 oligonucleotide radioactivity was 3.8×10^5 dpm/ml prior to methanol addition. Samples labeled P contained a final concentration of 5×10^{-4} M puromycin, those labeled O contained 5×10^{-4} M ψ-hydroxypuromycin, and those labeled B (blank) contained no puromycin or ψ-hydroxypuromycin. Samples were incubated 2.5 hr at 0°, then the reaction was terminated and the samples were 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 ethanol solutions were incubated in 1 M triethylamine for 30 min at 37°, 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 labeled U is [^{14}C]uridine used to locate the origin, corrected for the endosmotic effect. The arrows indicate the position of the ψ-hydroxypuromycin product.

as the dimethylamino group assumes a higher charge. The ψ-hydroxypuromycin product, indicated by arrows in Figure 5, has the same properties and this suggests that it also contains the nucleoside moiety of ψ-hydroxypuromycin. The products of the reaction with whole fMet-tRNA, directed by phage R17 RNA behave identically with those shown in Figure 5,

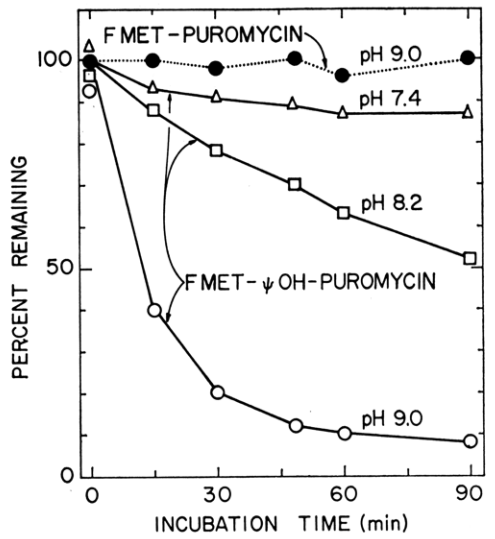

FIGURE 6: Alkaline hydrolysis of fMet-ψ-hydroxypuromycin. Products formed from [^{35}S]fMet-tRNA in the R17 RNA-stimulated system, as described under Methods. The concentration of fMet-tRNA was 9.3 A_{260} units/ml and the concentration of puromycin or ψ-hydroxypuromycin was 5×10^{-4} M. The ethyl acetate layer was 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 given and incubated at 30°. Aliquots were removed at various times for assay as described under Methods. Dotted line: fMet-puromycin. Solid lines: fMet-ψ-hydroxypuromycin.

except that the fMet-methyl ester present among the fragment system products is not formed in that system.

The product of the reaction between the oligonucleotide fragment or fMet-tRNA and ψ-hydroxypuromycin is shown to be an ester by its sensitivity to alkali. As shown in Figure 5e, fMet-ψ-hydroxypuromycin is destroyed by 1 M triethylamine with release of *N*-formylmethionine. In this respect it differs from fMet-puromycin which is stable under these conditions because the bond between fMet and puromycin is an amide. fMet-ψ-hydroxypuromycin is also hydrolyzed under relatively mild alkaline conditions as shown in Figure 6. While fMet-puromycin is unaffected at pH 9.0, fMet-ψ-

TABLE II: Reaction between fMet-tRNA and ψ-Hydroxypuromycin.

	dpm
Complete system[a]	796
Minus ribosomes	4
Minus ribosomes plus post-ribosomal supernatant (60 μg of protein)	−7
Plus 1 mM chloramphenicol	9
Plus 1 mM gougerotin	−4

[a] The complete reaction mixture is described under Methods (fMet–tRNA reaction). Blanks (minus ψ-hydroxypuromycin) ranging from 40 to 73 dpm have been subtracted.

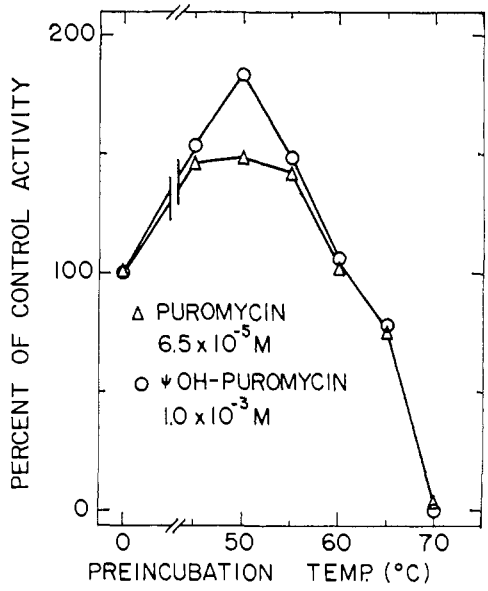

FIGURE 7: Heat inactivation of ribosomes. Ribosomes (13.8 A_{260} units/ml) were incubated in 0.4 M KCl, 0.02 M magnesium acetate, and 0.06 M Tris-HCl (pH 8.1 at 0°) for 5 min at various temperatures, then kept at 0° for 15 min. [^{35}S]fMet T_1 oligonucleotide (50,000 dpm/ml) was added and the mixture was divided into 0.1-ml aliquots. To each aliquot was added 0.1 ml of 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° the reaction was terminated and product was 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° throughout (control) after subtraction of the appropriate blank. The control values were 644 dpm for puromycin and 174 dpm for ψ-hydroxypuromycin.

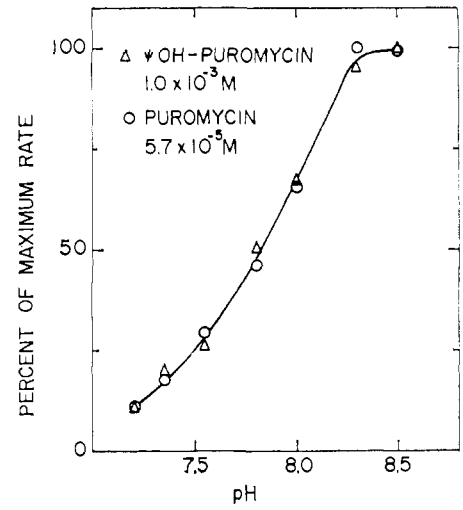

FIGURE 8: Effect of pH on the rate of reaction between fMet T_1 oligonucleotide and ψ-hydroxypuromycin or puromycin. Prior to addition of methanol, 0.1 ml of reaction mixtures contained: ribosomes (1.38 A_{260} units), fMet T_1 oligonucleotide (5900 dpm), 0.4 M KCl, 0.02 M magnesium acetate, and 0.06 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate buffer. The pH was determined at 0° in a similar mixture without ribosomes and the T_1 oligonucleotide. The reaction was initiated by adding 0.1 ml of 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°, 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 per cent of the pH 8.5 values, which were 368 dpm for puromycin and 175 dpm for ψ-hydroxypuromycin.

hydroxypuromycin begins to be hydrolyzed appreciably at a pH just over 8, and is destroyed rapidly at pH 9.0. Similar lability is observed for the fragment system product as well as the product formed from whole fMet-tRNA. At pH 9.0, 30° in 0.04 M Tris-HCl, fMet-ψ-hydroxypuromycin has a half-life of 12 min. For comparison, the fMet-methyl ester formed in the fragment system has a half-life of 130 min and formylmethionyladenosine, formed by pancreatic RNase digestion of fMet-tRNA, has a half-life of 3 min under these conditions.

We have concluded that this ester bond involves the α-hydroxyl group of ψ-hydroxypuromycin (Figure 1) rather than the 2'- or 5'-hydroxyls. Other analogs of puromycin in which the α-amino group is N acetylated or replaced by a chlorine atom or methoxy group are not reactive under these conditions even though the 2'- and 5'-hydroxyls are unaltered in these compounds. For example, *N*-acetylpuromycin is inactive in the fragment system at concentrations up to 2×10^{-2} M, even though it is a competitive inhibitor of the puromycin reaction, with $K_i = 6 \times 10^{-3}$ M. Since it is unable to react at concentrations where it is able to bind to the active site of the enzyme, its inactivity is not due solely to lack of affinity for the enzyme.

Role of the Ribosome. The properties shown in Tables I and II suggest that the reactions of ψ-hydroxypuromycin and hydroxypuromycin are catalyzed by the same enzyme which catalyzes the puromycin reaction, *i.e.*, the ribosomal peptidyl transferase. Further similarity of these reactions is shown by the heat inactivation data of Figure 7. Ribosomes were preincubated for 5 min at various temperatures, then tested for their activity in a subsequent incubation in the fragment system at 0°. Preincubation of the ribosomes at temperatures above 60° results in a parallel loss of their ability to catalzye fMet-puromycin and fMet-ψ-hydroxypuromycin formation. At temperatures below 60° a slight activation of the ribosomes is observed. Similar ribosomal activation has been observed by Bodley (1969) and Miskin *et al.* (1968).

Maden and Monro (1968) have shown that the activity of the ribosomal peptidyl transferase increases with increasing pH between 7.0 and 8.5. A similar pH dependence is observed for the reaction with ψ-hydroxypuromycin, as shown in Figure 8. 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 pointed out, the pH dependence for the puromycin reaction might alternatively be due to protonation of the α-amino group of puromycin if its pK is altered slightly on complex formation. This ambiguity is now eliminated 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 an N-terminal α-amino group might be involved in the catalysis of the reaction.

The hydroxyl analogs of puromycin are also active in bringing about the release of polypeptide chains. In an *E.*

coli in vitro system synthesizing polyphenylalanine directed by polyuridylic acid, hydroxypuromycin is able to bring about the release of oligophenylalanine into the supernatant. Like puromycin, hydroxypuromycin is also able to stop the incorporation of radioactive amino acids into a culture of *E. coli* although higher concentrations are required. The activity of hydroxypuromycin is thus similar to puromycin even though it is somewhat less effective, as one would anticipate in view of the reduced binding constant and velocity maximum shown in Figure 4.

Discussion

The hydroxyl analogs of puromycin are responsible both in the fragment reaction and in the R17 RNA system for the ribosome-catalyzed formation of a compound which is electrophoretically indistinguishable from formylmethionylpuromycin. This product differs from formylmethionylpuromycin by its alkaline lability, indicative of an ester bond between fMet and hydroxypuromycin. 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 hydroxypuromycin and puromycin reactions undergo parallel inactivation at high temperatures. (5) The reaction is specific for the L isomer of ψ-hydroxypuromycin.

The involvement of the α-hydroxyl group of hydroxypuromycin in the reaction is indicated by the fact that other analogs of puromycin with inert groups at this position are inactive. Because of the electrophoretic similarity of the product of fMet-puromycin and the alkaline lability of the bond between formylmethionine and hydroxypuromycin we have concluded that the structure of the product is as shown in Figure 1.

The capability of peptidyl transferase for ester formation indicates a flexibility in the catalytic mechanism; however, it does not necessarily imply a special function of the enzyme *in vivo*. Nevertheless, the fact that the ribosome has this capacity opens the possibility that ester bonds could be inserted into polypeptides. Whether or not this occurs *in vivo* depends upon whether the appropriate substrates exist in the cell and, if they do, whether they can gain access to the enzyme.

This flexibility might be related to the involvement of peptidyl transferase in polypeptide chain termination, which is supported by evidence presented by Vogel *et al.* (1969). The ability of the enzyme to transfer the nascent polypeptide to a hydroxyl group is required if the enzyme is to catalyze the hydrolytic release of the nascent peptide from tRNA. In that case the acceptor would be the hydroxyl group of water and, as suggested by Vogel *et al.* (1969), a release factor might change the specificity of the enzyme so that water can take the place of the usual adenosine derivative. Thus the reaction with hydroxypuromycin might represent a hybrid case, where the acceptor is a hydroxyl group as in chain termination, but the accepting group is part of an acyladenosine derivative, as in peptide bond formation.

The ribosomal peptidyl transferase is a central enzyme in molecular biology: it is responsible for the synthesis of all proteins. It is therefore of considerable interest to learn the mechanism of its action. The present paper demonstrates its ability to catalyze the formation of an ester bond as well as its normal product, the peptide bond. A similar flexibility is observed with peptidases, most of which can catalyze the hydrolysis of ester bonds as well as peptide bond (Dixon and Webb, 1964). A further similarity to the serine proteases is indicated by the pH dependence of peptidyl transferase reaction. This may reflect a basic similarity of the transition states for both the synthetic and the hydrolytic enzymes.

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