Peptidylpuromycin Formation on Mammalian Polysomes. Studies on Transpeptidation and Translocation†

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ABSTRACT: Rat liver polysomes free of supernatant factors retain their nascent polypeptide chains. Some of these chains react with puromycin without the addition of a soluble factor. Others require translocation, catalyzed by elongation factor 2, before they react. Thus, rat liver polysomes can be used, without the addition of exogenous peptidyl- or aminoacyl-tRNA, for investigations of both transpeptidation and translocation. Incorporation of [^{3}H]puromycin into hot trichloroacetic acid precipitable material is taken as a measure of peptidylpuromycin formation. Some precipitable [^{3}H]puromycin remains bound to ribosomes. Experiments with ribosomes containing nascent ^{14}C-labeled polypeptide chains show that nascent chains as well as [^{3}H]puromycin are bound. Presumably some peptidylpuromycin is bound through interactions other than hydrogen bonding between the codon of mRNA and the anticodon tRNA. The extent of the reaction with puromycin is proportional to the number of ribosomes present, but it is also a function of the concentration of both puromycin and elongation factor 2. Under the conditions used here, the total amount of peptidylpuromycin is probably a reflection of the initial rate rather than a measure of ribosomes with reactive peptide chains.

Kinetic experiments with inhibitors of transpeptidation in an assay uncomplicated by factors affecting the binding of exogenous peptidyl- or aminoacyl-tRNA indicate that anisomycin, sparsomycin, neomycin, and ethanol are competitive with puromycin while gougerotin, cycloheximide, and fusidic acid are not competitive.

Puromycin has been used extensively as an analog of aminoacyl-tRNA, for investigating transpeptidation and translocation in protein synthesis. The conclusion that transpeptidation is catalyzed by an integral part of the large ribosomal subunit in both prokaryotic and eukaryotic organisms is based largely on studies with puromycin. Small aminoacyl-terminal fragments of aminoacyl-tRNA, as well as intact aminoacyl-tRNA, react to form aminoacylpuromycin in the presence of ethanol and isolated 50S or 60S ribosomal subunits (Monro, 1967; Monro *et al.*, 1969; Vazquez *et al.*, 1969). Under more physiological conditions, exogenous peptidyl-tRNA (Traut and Monro, 1964; Rychlik, 1966; Gottesman, 1967), formylmethionyl-tRNA (Bretscher and Marcker, 1966; Zamir *et al.*, 1966), and *N*-acetylphenylalanyl-tRNA (Lucas-Lenard and Lipmann, 1966; Weissbach *et al.*, 1968) react to form peptidyl- or aminoacylpuromycin in the presence of washed 70S or 80S ribosomes. According to the two-site model for ribosomes and the current concept of the mechanism of action of puromycin (Lipmann, 1969; Lengyel and Soll, 1969; Pestka, 1971a), those peptidyl- or aminoacyl-tRNAs which react with puromycin are bound in the peptidyl or donor site on the ribosome.

Translocation, the GTP-dependent process in which a newly lengthened peptidyl-tRNA moves from the acceptor to donor site on the ribosome, can be coupled to transpeptidation and investigated by reaction with puromycin. Bacterial EF-G (G factor) and mammalian EF-2 (aminoacyltransferase II, T_2)[1] facilitate the conversion of peptidyl-tRNA from an unreactive condition on the ribosome to one in which reaction with puromycin occurs. Facilitation of this conversion by EF-G (Tanaka *et al.*, 1968; Brot *et al.*, 1968; Kaji *et al.*, 1969; Skoultchi *et al.*, 1969) and EF-2 (Skogerson and Moldave, 1968; Schneider *et al.*, 1968; Hardesty *et al.*, 1969) constitutes a part of the evidence that these factors catalyze translocation.

Interpretation of experiments using puromycin to study either peptide-bond formation *per se* or translocation coupled to peptide-bond formation are often complicated by factors which influence the binding of exogenous peptidyl- or aminoacyl-tRNA to ribosomes. These complications can be avoided by the use of ribosomes which retain their nascent polypeptide chains. Rat liver polysomes are well suited for such experiments, since they retain nascent chains even after removal of bound supernatant factors. Some polypeptide chains react with low concentrations of puromycin without the addition of a supernatant factor, and are thus presumed to be in the donor site. Others require the addition of EF-2 and GTP in order to form peptidylpuromycin, and are thus presumed to be in the acceptor site (Skogerson and Moldave, 1968; Schneider *et al.*, 1968).

Previously, we have shown that the inactivation of EF-2 in partially purified supernatant fractions by diphtheria toxin and NAD^+ abolishes the supernatant factor GTP-dependent increase in polypeptide chains reactive with puromycin (Schneider *et al.*, 1968). We have purified EF-2 to homogeneity and found that the ability to facilitate the reaction with puromycin copurifies with other known properties of EF-2 (Raeburn *et al.*, 1971). In the present paper some general characteristics of the reaction of nascent polypeptide chains with [^{3}H]-puromycin in the presence or absence of EF-2 are presented. The data support the validity of the polysome–puromycin system for investigating both transpeptidation and trans-

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[1] In the uniform nomenclature recently proposed for the factors involved in protein synthesis (Caskey *et al.*, 1972) aminoacyltransferase I was designated EF-1 for elongation factor 1, aminoacyltransferase II, EF-2, for elongation factor 2 and bacterial factor G, EF-G, for elongation factor G. ADPR is the adenosine diphosphate ribose moiety of NAD^+.

TABLE I: Removal of EF-2 from Ribosomes by Washing with High Salt.[a]

Ribosomes		Molar Ratio of Compound Incorporated to Ribosomes Present				
			[^{14}C]Leucine		[^{3}H]Puromycin	
Washes with 0.5 M NH_4Cl	RNA: Protein	[^{3}H]ADPR	Minus EF-2	Plus EF-2	Minus EF-2	Plus EF-2
None	0.73	0.03	0.06	0.13	0.04	0.07
One	0.95	<0.001	<0.003	0.07	0.04	0.09
Two	1.10	<0.001	<0.003	0.06	0.04	0.09

[a] Preparation of ribosomes and assays for incorporation of labeled compounds into hot trichloroacetic acid precipitable material are described in Materials and Methods. Reaction mixtures contained: for ADPR incorporation from [^{3}H]-NAD^+, 500–1000 pmol of ribosomes; for leucine or puromycin incorporation, 50–100 pmol of ribosomes and 0.1 mg of total protein of EF-2 were indicated. A molecular weight of 4×10^6 was assumed for rat liver ribosomes. The specific activities of the labeled compounds were: [^{3}H]NAD^+, 33 cpm/pmol; [^{3}H]puromycin, 720 cpm/pmol; and [^{14}C]leucine, 200 cpm/pmol. The times and temperatures of incubations were 60 min at 22° for [^{3}H]ADPR or [^{3}H]puromycin incorporation and 10 min at 37° for [^{14}C]leucine incorporation.

location. Results of kinetic studies on the effect of several inhibitors of protein synthesis on transpeptidation are included.

Materials and Methods

Materials. [*methoxy*-^{3}H]Puromycin dihydrochloride (1.1 Ci/mmol) [^{14}C] amino acid mixture and L-[^{14}C]leucyl-tRNA (0.355 μCi of [^{14}C]leucine/mg of aminoacyl-tRNA) were from New England Nuclear. [^{3}H]NAD^+ labeled in the adenosine moiety was prepared as described previously (Goor and Maxwell, 1970) from Li_4[8-^{3}H]ATP (24.5 Ci/mmol) from Schwarz BioResearch. Puromycin dihydrochloride and the aminonucleoside derived from puromycin were purchased from Nutritional Biochemicals; unstripped aminoacyl-tRNA (rat liver) and stripped tRNA (*Escherichia coli*) from General Biochemicals; cycloheximide, phosphoenolpyruvate, and pyruvate kinase from Sigma; and neomycin from Mann. Anisomycin, a product of Charles Pfizer, was supplied by Mr. Nathan Belcher. Gougerotin was a gift from Dr. Jack Fox, Sloan Kettering Institute for Cancer Research, New York, N. Y. Fusidic acid, a product of Squibb, was obtained through Mr. J. Pratt of Squibb or from the National Cancer Institute Drug Development Branch, Bethesda, Md. Sparsomycin, a product of Upjohn, was obtained from the National Cancer Institute Drug Development Branch.

Diphtheria toxin was purified as described previously (Goor and Pappenheimer, 1967).

Ribosomes were prepared from rat liver by the method of Wettstein *et al.* (1963) and were further purified by two centrifugations through discontinuous sucrose gradients containing 0.5 M NH_4Cl (Skogerson and Moldave, 1968).

EF-2 was partially purified from rat liver and resolved from EF-1 as described previously (Gasior and Moldave, 1965).

Assays. EF-2 was analyzed by ADP ribosylation with [^{3}H]NAD^+ and diphtheria toxin under conditions which assure complete reaction of EF-2 (Raeburn *et al.*, 1971).

The transfer of [^{14}C]leucine from [^{14}C]leucyl-tRNA into protein was carried out as described (Raeburn *et al.*, 1971).

For the formation of peptidylpuromycin, ribosomes were reacted with [^{3}H]puromycin in a final volume of 0.5 ml containing 6 mM $MgCl_2$, 320 mM NH_4Cl, and 50 mM Tris buffer, pH 7.3 at 25°. Unless otherwise specified the concentration of puromycin was 1.8 μM. When EF-2 was present, 0.35 mM GTP and 30 mM β-mercaptoethanol were added. The reaction was stopped with 5 ml of 5% trichloroacetic acid, carrier protein was added when necessary, and the precipitates were collected and prepared for counting as described (Schneider *et al.*, 1968) except that 10 μl of 12 N H_2SO_4 was added following solubilization of the protein with alkaline Nuclear-Chicago Solubilizer.

Protein was determined by the method of Lowry *et al.* (1951). rRNA was estimated by absorbance at 260 nm.

Results and Discussion

Characteristics of Polysomes. Polysomes are most useful for the study of transpeptidation and of translocation coupled to transpeptidation if they can be isolated with their nascent polypeptide chains intact, and free of EF-2. The data in Table I confirm by two criteria that EF-2 is removed from polysomes by 0.5 M NH_4Cl and show that some nascent chains are retained. Diphtheria toxin catalyzes the incorporation of the adenosine diphosphate ribose moiety of NAD^+ into a covalent linkage with EF-2 (Honjo *et al.*, 1968, 1971; Gill *et al.*, 1969); 1 mol of ADPR is incorporated per mol of EF-2 (Raeburn *et al.*, 1971; Collins *et al.*, 1971; Robinson and Maxwell, 1973). The data in Table I indicate that 3% of the unwashed ribosomes contained one molecule of bound EF-2, or alternatively that a smaller number of ribosomes had more than one bound molecule of EF-2. No detectable EF-2 remained after one wash with 0.5 M NH_4Cl. EF-2 was also assayed by its ability to complement EF-1 for the incorporation of [^{14}C]-leucine from [^{14}C]leucyl-tRNA into protein. The data in Table I show that unwashed polysomes incorporated [^{14}C]-leucine into their nascent polypeptide chains without the addition of EF-2. After one wash with 0.5 M NH_4Cl no detectable incorporation of leucine occurred in the absence of exogenous EF-2.

The remaining data in Table I show that at least 4% of the ribosomes contained nascent polypeptide chains, presumably in the donor site, which reacted with puromycin in the absence of EF-2. The addition of EF-2 increased the number of ribosomes with reactive chains to between 7 and 9%. These data can be interpreted to indicate that a minimum of 3–5% of the ribosomes contained nascent chains in the acceptor site and that translocation catalyzed by EF-2 was required to render them reactive with puromycin. This interpretation is supported by two observations. First, the soluble factor that facilitates stimulation of the puromycin reaction copurifies to homogeneity with EF-2 assayed independently by other methods (Raeburn *et al.*, 1971); and second, the soluble factor-dependent stimulation of the puromycin reaction is inhibited by diphtheria toxin together with NAD^+ (Schneider *et al.*, 1968), a specific inhibitor of EF-2. In this experiment (Table I) an effect of EF-2 was apparent even with unwashed ribosomes. In other experiments it was necessary to wash ribosomes with 0.5 M NH_4Cl in order to observe a stimulation of the reaction with puromycin by exogenous EF-2. The total number of ribosomes capable of donating polypeptide chains

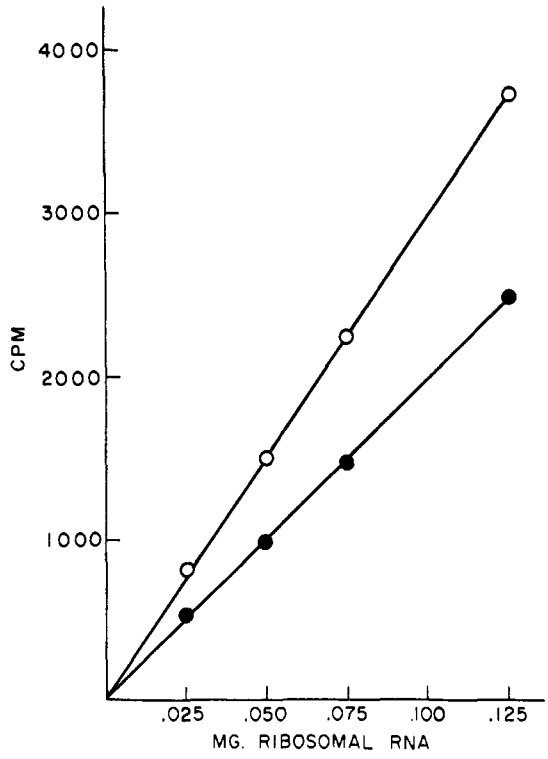

FIGURE 1: Peptidyl[^{3}H]puromycin formation with varying amounts of ribosomes. The reaction was performed at 37° for 90 min as described in Materials and Methods with (open circles) or without (closed circles) GTP, mercaptoethanol, and EF-2. The EF-2 in each incubation contained 0.4 mg total protein.

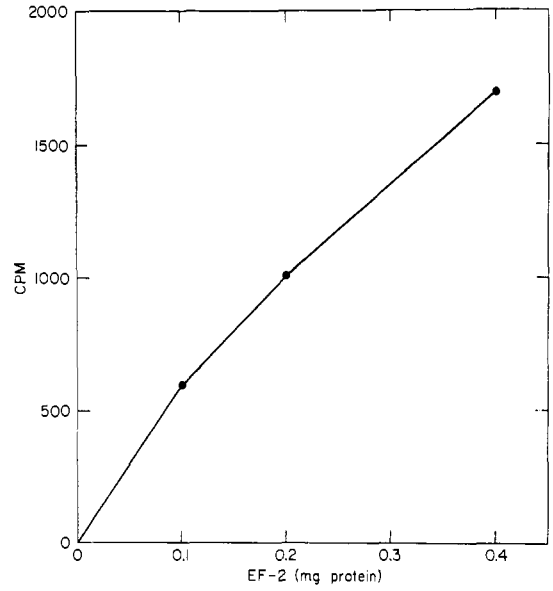

FIGURE 2: EF-2 stimulated increase in total peptidyl[^{3}H]puromycin formation as a function of EF-2 concentration. The reaction was performed at 37° for 60 min as described in Materials and Methods. The difference between the values obtained with and without EF-2, GTP, and mercaptoethanol is plotted as a function of EF-2 concentration. The ribosomes in each assay contained 33 μg total protein.

to form peptidylpuromycin, under the conditions of the assay employed, varied from experiment to experiment in the range of 8–20%.

General Characteristics of the Reaction. The reaction with puromycin in the absence of EF-2 requires Mg^{2+} and NH_4^+ and is not significantly changed by mercaptoethanol or GTP (Table II). These data document previous statements (Skogerson and Moldave, 1968; Schneider *et al.*, 1968), and are in accord with requirements for transpeptidation in other systems. Stimulation of the reaction by soluble protein is effected by mercaptoethanol and GTP, both known to be required for translocation in other systems. In this assay, as in others for EF-2, GTP cannot be replaced by the β,γ-methylene analog of GTP, GDP, GMP, ATP, or cAMP.

The extent of incorporation of [^{3}H]puromycin into hot trichloroacetic acid precipitable material, both in the presence or absence of EF-2, is proportional to the number of ribosomes present (Figure 1). Assuming that rat liver ribosomes are 50% protein and have a molecular weight of 4×10^6 (Wettstein *et al.*, 1963) about 11% of the ribosomes in this experiment contributed a polypeptide chain to puromycin without exogenous EF-2, and an additional 7% reacted when the soluble factor was added.

It has been shown previously that the initial rate of the EF-2 stimulated reaction with puromycin is a function of the amount of EF-2 added. An assay based on the increased rate of reaction brought about by the addition of supernatant factors was used in our studies to demonstrate that the soluble factor which facilitates the puromycin reaction copurifies with EF-2 assayed independently by three other reactions (Raeburn *et al.*, 1971). As shown in Figure 2, the extent of the EF-2-facilitated reaction also depends on EF-2 concentration. The addition of more EF-2 after the reaction has stopped does not induce further reaction. It is reasonable that the initial rate should be dependent of EF-2 concentration, but it is not clear why the extent of the reaction as well is a function of EF-2 concentration.

The extent of the reaction depends also on the concentration of puromycin (Figure 3). Skogerson and Moldave (1968) suggested that nascent chains in the acceptor site in addition to those in the donor site might react if high concentrations of puromycin are used. The data in Figure 3 could be interpreted in this way, but alternative explanations are possible. For example, the initial rate of transpeptidation would be de-

TABLE II: Requirements for Transpeptidation and Translocation.[a]

System	[^{3}H]Puromycin with EF-2 (cpm)	Incorporated without EF-2 (cpm)
Complete	4640	2880
$-Mg^{2+}$	2290	753
$-NH_4^+$	2010	1080
−Mercaptoethanol	3730	2710
−GTP	3390	2440

[a] Reaction with [^{3}H]puromycin and preparation of samples for counting were carried out as described in Materials and Methods. Incubation was for 40 min at 22° with ribosomes (0.1 mg of protein) and EF-2 (0.4 mg of protein) where indicated. Concentrations of 0.5 mM Mg^{2+} and 2.5 mM K^+ were contributed by buffer added with the ribosomes. EF-2 (25 μl) was added in 50 mM Tris buffer containing 1 mM dithiothreitol and 0.1 mM EDTA.

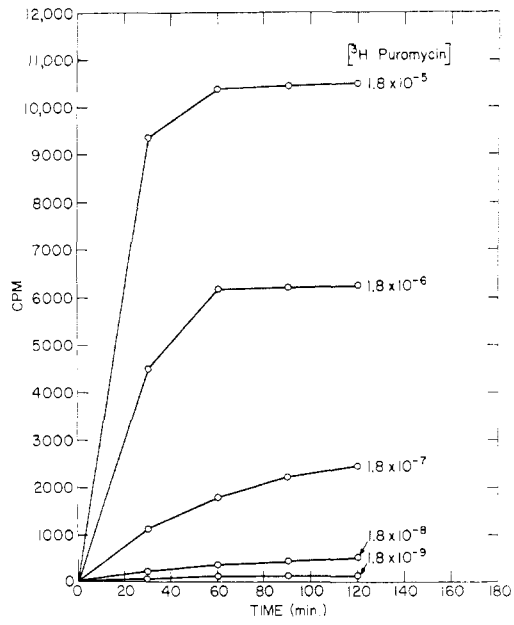

FIGURE 3: Peptidyl[^{3}H]puromycin formation at varying concentrations of puromycin. The reaction was performed at 37° for the times indicated as described in Materials and Methods. The ribosomes in each assay contained 90 μg total protein. EF-2, GTP, and mercaptoethanol were not added.

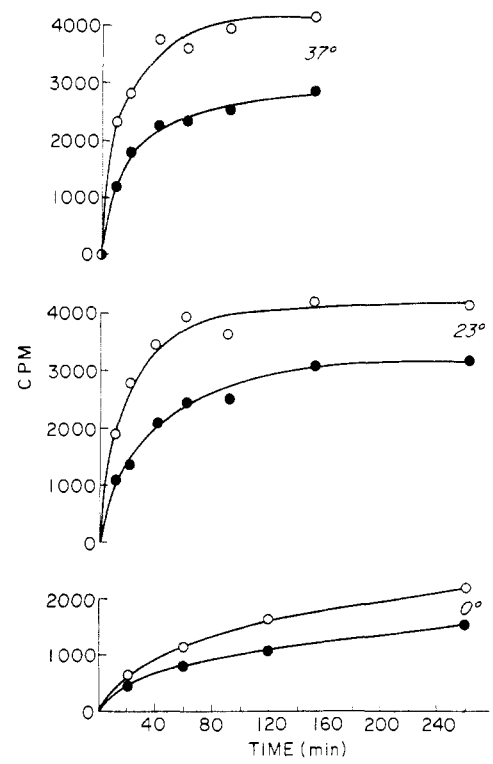

FIGURE 4: Peptidyl[^{3}H]puromycin formation at 0, 23, and 37°. The reaction was performed at the temperature and for the times indicated, as described in Materials and Methods, either with (open circles) or without (closed circles) GTP, mercaptoethanol, and EF-2. The EF-2 in each incubation contained 0.4 mg total protein. The ribosomes in each assay contained 75 μg total protein.

pendent on puromycin concentration at concentrations below the K_m for the reaction. If some component in the system becomes inactivated during the incubation, the reaction might never reach completion. If this is the case, the extent of the reaction is not directly a measure of the total number of available reactive polypeptide chains, but is rather a reflection of the initial rate of the reaction. The K_m for puromycin in transpeptidation on rat liver polyribosomes based on initial rates determined under somewhat different conditions from those used in these experiments is 8×10^{-6} M (Pestka *et al.*, 1972). Calculation of a "K_m" for puromycin substituting the final values in Figure 3 for initial rates gives a comparable value of 2×10^{-5} M. The initial rate, and in this case the final value, would be influenced by the concentration of translocated peptide chains as well as by that of puromycin. This interpretation is consistent with the observation described above (Figure 2) that the extent of the reaction is a function of EF-2 concentration.

Preliminary experiments indicated that reaction with puromycin occurred even at 0°. The experiments depicted in Figure 4 were undertaken to see if peptide-bond formation might be studied in crude systems without the occurrence of translocation. The data show that both reactions take place at low temperatures and indicate that purified ribosomes are preferable for dissociation of transpeptidation from translocation.

It is well established that puromycin releases nascent polypeptide chains from ribosomes. If polypeptidyl-tRNA is bound only through the codon–anticodon interaction all chains that react with puromycin should be released. Allen and Zamecnik (1962) found that concentrations as high as 600 μM released only 20–30% of labeled polypeptide chains from rabbit reticulocyte ribosomes. Following incubation of unlabeled ribosomes with [^{14}C]puromycin the same investigators observed that about 65% of hot trichloroacetic acid precipitable puromycin remained bound to ribosomes. The recovery of hot trichloroacetic acid precipitable ^{14}C label in the ribosomal pellet was not altered by treatment with RNase but was eliminated by treatment with proteolytic enzymes. Allen and Zamecnik concluded that puromycin bound to the ribosome probably was in peptide bond with polypeptide chains adsorbed to the ribosome through interactions other than hydrogen bonding between codon and anticodon.

In most of the experiments reported here and in previous work (Skogerson and Moldave, 1968; Schneider *et al.*, 1968), the ribosomal pellet was not separated from the supernatant fluid and the total hot trichloroacetic acid precipitable [^{3}H]-puromycin was taken as a measure of the reaction of puromycin with polypeptidyl-tRNA to form polypeptidylpuromycin. If puromycin reacts with ribosomes in some other way the method is not valid. The experiments depicted in Figures 5 and 6 were designed to investigate the possibility of spurious reaction of puromycin with polysomes.

The data in Figure 5 show the distribution between ribosomal pellets and supernatant fractions of nascent polypeptide chains, labeled *in vivo* with [^{14}C]amino acids, and hot trichloroacetic acid precipitable [^{3}H]puromycin after reaction of rat liver polysomes with three different concentrations of [^{3}H]puromycin in the presence or absence of EF-2. The distribution of ^{14}C was not significantly altered by puromycin concentration over the fourfold range used here. The total amount of hot trichloroacetic acid precipitable [^{3}H]puromycin increased with puromycin concentration, but the distribution was not changed. At each concentration of puromycin, the total trichloroacetic acid precipitable ^{3}H label was increased by EF-2, and the increase was equally apparent in the ribosomal pellet and in the released material. In all cases, the amount of [^{14}C]polypeptide remaining on the ribosomes appears to be

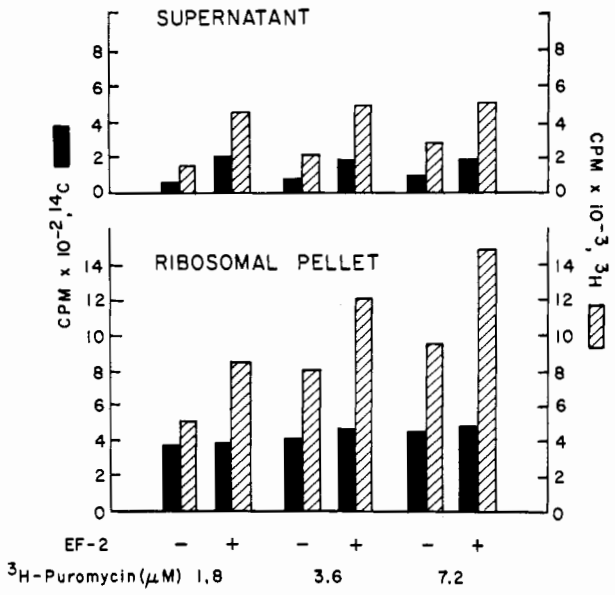

FIGURE 5: The distribution of nascent polypeptide chains, labeled *in vivo* with [^{14}C]amino acids, and hot trichloroacetic acid precipitable [^{3}H]puromycin after reaction of rat liver polysomes with three different concentrations of [^{3}H]puromycin in the presence or absence of EF-2. [^{14}C]Amino acid mixture (25 μCi) was adjusted to pH 7.2 and injected in a volume of 1 ml into the portal vein of a 100-g Sprague-Dawley male rat. After 1 min, the rat was sacrificed and the liver ribosomes were prepared as described in Materials and Methods. The incubations with [^{3}H]puromycin were performed at 37° for 60 min as described in Materials and Methods. Each reaction mixture was then layered on 0.5 ml of buffer medium (0.5 M sucrose, 50 mM Tris buffer (pH 7.8), 5 mM $MgCl_2$, and 36 mM KCl) and centrifuged at 105,000g for 2 hr. The ribosomal pellets were rinsed twice with buffer (as above except 0.25 M sucrose) without disturbing the pellet. The pellets were suspended in buffered sucrose (0.25 M) and recovered by centrifugation. The washings and supernatant fluids were combined, a 500-fold excess of unlabeled puromycin was added to both the pellet and supernatant fractions and they were incubated at 37° for 5 min. Trichloroacetic acid was added and the samples were prepared and counted as described in Materials and Methods.

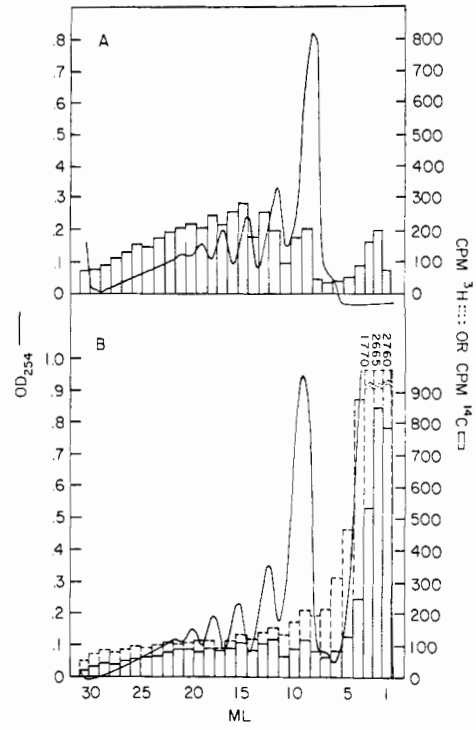

FIGURE 6: Centrifugation of ribosomes with ^{14}C-labeled nascent polypeptide chains on a sucrose gradient after incubation with EF-2 in the presence (B) and absence (A) of [^{3}H]puromycin. Rat liver ribosomes were labeled by incubation of 20 ml of postmitochondrial supernatant fraction with 100 μCi of [^{14}C]amino acid mixture, 10 mg of stripped tRNA (*E. coli*), 1 mg of pyruvate kinase, 0.25 nmol of phospho(enol)pyruvate, 5.25 μmol of GTP, and 38 μmol of ATP in a total volume of 30 ml for 1 hr at 22°. Following incubation, ribosomes were isolated as described in Materials and Methods, except that they were not washed with NH_4Cl. The isolated ^{14}C-labeled ribosomes were incubated at 22° for 60 min in the usual incubations with [^{3}H]puromycin and EF-2 (0.1 mg of protein). Puromycin was omitted in the experiment illustrated in part A. Following the incubation, each sample was layered on a linear 10–34% sucrose gradient (w/v, total volume 27 ml) containing 50 mM Tris buffer (pH 7.3), 25 mM KCl, and 5 mM $MgCl_2$. The tubes were centrifuged in a Spinco SW-25.1 rotor at 25,000 rpm for 3 hr. Fractions were collected by upward displacement in an Isco Model D density gradient fractionator with a Model 222 uv analyzer and recorder. Trichloroacetic acid was added to each fraction and the precipitates were prepared and counted as described in Materials and Methods.

sufficient to account for [^{3}H]puromycin in peptide bond with nascent polypeptide chains. Treatment of a ribosomal pellet with RNase decreased both the [^{14}C]polypeptides and the [^{3}H]puromycin in hot trichloroacetic acid precipitates by about 50%, probably due to incomplete precipitation of short chains after disruption of the ribosomes. Treatment with pronase completely eliminated both ^{14}C and ^{3}H in the hot trichloroacetic acid precipitate.

Figure 6A shows the distribution following centrifugation in a sucrose gradient of ribosomes labeled *in vitro* with [^{14}C]-leucine. A small amount of radioactivity is in the supernatant area and the remainder is distributed throughout the ribosomal pattern with the ratio of optical density to [^{14}C]leucine greater in the monosomes and small polysomes. Figure 6B shows a similar pattern of ribosomes following reaction with [^{3}H]puromycin and EF-2. About 30% of [^{14}C]leucine was released from ribosomes and 80% of hot trichloroacetic acid precipitable [^{3}H]puromycin was found in the supernatant fractions (fractions 1–7).

The ratio of [^{3}H]puromycin to [^{14}C]leucine was higher in monosomes and heavy polysomes, but there was no indication of a spurious reaction whereby puromycin remained attached to ribosomes not containing nascent chains. Release of [^{14}C]-leucine occurred from monosomes as well as from all sizes of polysomes, but the per cent released increased from 23% in the monosome–disome region (fractions 8–14) to 42% in the heavy polysome area (fractions 24–31). This might indicate that longer peptidylpuromycin chains are more readily released from ribosomes.

Kinetic Studies of Transpeptidation. In our previous reports (Schneider *et al.*, 1968; Raeburn *et al.*, 1971), the reaction of nascent polypeptide chains with puromycin was utilized primarily for studying translocation catalyzed by EF-2. Advantages of the system apply equally to the study of peptide-bond formation *per se*. Thus, we studied the effects of some inhibitors of protein synthesis on transpeptidation. Recently, Pestka and his collaborators[2] (Pestka, 1972; Pestka *et al.*, 1972, 1973) have used a similar assay with both bacterial and mammalian ribosomes to elucidate some of the basic characteristics of transpeptidation. They have also studied the effect of inhibition of the process in much the same way as de-

[2] We thank Dr. Pestka for making his manuscripts available to us prior to publication.

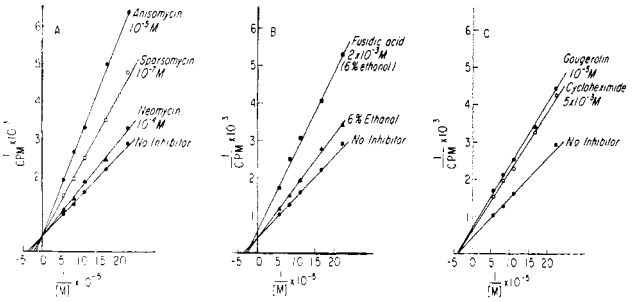

FIGURE 7: Reciprocal plots of peptidyl[^{3}H]puromycin formation *vs.* puromycin concentrations. The reactions were performed at 22° for 1 min as described in Materials and Methods. EF-2, GTP, and mercaptoethanol were not present. The reactions were started by the addition of ribosomes and terminated with trichloroacetic acid.

scribed here. Our present studies were carried out before we were aware of the superiority of K^+ over NH_4^+ (Pestka *et al.*, 1972) for the system, but preliminary evidence suggests that substitution of K^+ would not alter our general conclusions.

The reaction of nascent polypeptide chains to form peptidyl[^{3}H]puromycin in the absence of EF-2 is linear with time for at least 3 min, a period sufficiently long to permit kinetic studies. Experiments with selected inhibitors and varying concentrations of puromycin were carried out under conditions which assured linearity with respect to time. As shown in Figure 7, reciprocal plots of peptidyl[^{3}H]puromycin formation *vs.* puromycin concentration divide the inhibitors into two groups. Anisomycin, sparsomycin, neomycin, and ethanol are competitive with puromycin. Gougerotin and relatively high concentrations of cycloheximide and fusidic acid are not competitive. Similar results were obtained by Pestka *et al.* (1973) for anisomycin, sparsomycin, gougerotin, and cycloheximide in an assay essentially the same as that used here. The previously observed effects of these antibiotics on ribosomal function have been reviewed recently (Pestka, 1971a). Sparsomycin is a competitive inhibitor of puromycin-induced release of peptide chains from *E. coli* ribosomes (Goldberg and Mitsugi, 1967) and competes with puromycin in the formation of acetylphenylalanylpuromycin from acetylphenylalanyl-tRNA and puromycin (Pestka, 1970). Anisomycin inhibits transpeptidation as measured by the "fragment reaction" (Vazquez *et al.*, 1969; Neth *et al.*, 1970). Neomycin interacts with the 30S subunit in bacterial systems from sensitive organisms and causes extensive misreading of mRNA. In addition, it inhibits the puromycin-induced release of polylysine from tRNA (Cerna *et al.*, 1969) and inhibits [^{3}H]puromycin formation (Pestka, 1971b) on *E. coli* ribosomes. Ethanol also causes misreading of mRNA. Inhibition of transpeptidation by ethanol, as reported here, is of interest in view of the requirement for ethanol in the fragment reaction, another assay for the same process. Gougerotin inhibits the puromycin-stimulated release of nascent polypeptide chains from ribosomes (Casjens and Morris, 1965) and of $(Lys)_n$ from polylysyl-tRNA (Goldberg and Mitsugi, 1967; Pestka, 1970). In the latter case it exhibits mixed-type kinetics with puromycin. Several groups have reported that cycloheximide inhibits chain initiation and elongation (Pestka, 1971a). Its inhibition of chain elongation has been attributed to interference with translocation. Termination is also inhibited (Rajalakshmi *et al.*, 1971). Cycloheximide has not been reported to inhibit transpeptidation assayed by methods other than that used in these studies. Fusidic acid inhibits translocation by forming an unreactive complex with EF-2 (or EF-G) and GDP on the ribosome (Bodley *et al.*, 1970). The significance of the inhibition of transpeptidation observed in these studies is not clear.

No inhibition of transpeptidation was observed upon tbe addition of the aminonucleoside of puromycin, diphtheria toxin together with the NAD^+ or up to 100 μg of aminoacyl-tRNA. The latter observation is in accord with previous experiments (Coutsogeorgopoulos, 1967) which showed that the inhibition by puromycin of poly(uridylic acid)-directed $(Phe)_n$ synthesis was not reversed by increasing concentrations of phenylalanyl-tRNA.

Concluding Remarks

Many assays for transpeptidation and translocation make use of the reaction of puromycin with exogenous peptidyl- or aminoacyl-tRNA in the presence of ribosomes. These assays are affected by factors which influence the binding of the exogenous substrate to the ribosomes, but which have no direct effect on the actual processes of translocation and peptide-bond formation. The assays described here eliminate these indirect effects by utilizing the reaction of nascent polypeptide chains on purified ribosomes with [^{3}H]puromycin. At least two questions regarding the validity of tbe assays have arisen. First, since all trichloroacetic acid precipitable [^{3}H]puromycin is not released from ribosomes, a possibility exists that some reaction with puromycin other than that of nascent chains to form peptidyl[^{3}H]puromycin occurs. The data presented here do not prove that all trichloroacetic acid precipitable [^{3}H]-puromycin is in peptide bond with nascent polypeptide chains, but they do lend additional support to this concept. Experiments with polysomes containing nascent chains labeled with [^{14}C]leucine showed that in every case where acid-precipitable [^{3}H]puromycin was associated with ribosomes, some nascent chains remained bound in the same population of ribosomes. If the bound puromycin is in peptide bond with polypeptide chains, the chains can no longer be bound to ribosomes through the codon–anticodon interaction. The nature of the binding remains to be investigated, but the fact that a portion of both nascent chains and puromycin remains associated with ribosomes should be taken into account in the interpretation of experiments utilizing puromycin for the release of nascent chains.

The second question regarding the use of [^{3}H]puromycin to study transpeptidation and translocation on rat liver polysomes is concerned with the specificity of puromycin for nascent polypeptide chains in the donor site of the ribosome. Skogerson and Moldave (1968), on the basis of differences observed with different concentrations of puromycin, suggested that the reaction might be specific for the donor site at low, but not at high, concentrations. The data presented here support an alternative explanation, already mentioned, for the effect of puromycin concentration on the extent of the reaction. This alternative explanation is consistent with the generally accepted concept that reaction with puromycin occurs only from the donor site on the ribosome. Our hypothesis is that the reaction of nascent chains on rat liver polyribosomes with puromycin does not reach completion and that the final amount of peptidyl[^{3}H]puromycin formed is a reflection of the initial rate of the reaction. At low concentrations of puromycin the initial rate, and thus the final value, is a function of the concentration of puromycin. Although this does not invalidate the use of [^{3}H]puromycin to study transpeptidation and translocation, it should be pointed out that

even when puromycin is present in considerable excess the amount of peptidylpuromycin formed may not be a measure of the total number of active ribosomes with nascent polypeptide chains.

Results of our kinetic studies on transpeptidation and inhibition of the process are in general agreement with those of Pestka *et al.* (1972, 1973) and support the view that such kinetic studies are useful in elucidating the basic mechanism of peptide-bond formation and in studying factors which control this process.

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