# The Possible Involvement of Peptidyl Transferase in the Termination Step of Protein Biosynthesis*

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ABSTRACT: The ability of a cell-free ribosomal system to release nascent peptide in the presence of a termination codon and release factor has been found to run parallel with the ability of the 50S ribosomal subunit to synthesize peptide bonds (peptidyl transferase activity). The evidence is of two kinds.

(a) Use was made of the recent-finding that the peptidyl transferase activity of the 50S subunit can be abolished and subsequently restored by relatively mild treatments. We have found that the release reaction responds similarly: that is, inactivation of the 50S subunit eliminated both activities; reactivation restored both, and partial reactivation restored both partially and to the same degree. (b) A number of antibiotics that inhibited the peptidyl transferase activity of the 50S subunit also inhibited the release activity, and with the same dependence on antibiotic concentration. These results indicate that a component of the 50S ribosomal subunit that participates in the peptide-bond-forming step of chain extension may also participate in the release step of chain termination. They suggest, but do not unequivocally prove, that this component is the ribosomal enzyme peptidyl transferase, operating with an altered specificity, so as to transfer nascent peptide to water instead of to aminoacyl transfer ribonucleic acid.

When the synthesis of a polypeptide chain has been completed on a ribosome, the chain is detached from both the ribosome and the molecule of tRNA which held it on the ribosome. This event is signaled by a termination codon of the mRNA (Stretton *et al.*, 1966; Weigert *et al.*, 1966) and is mediated by a nonribosomal factor (Ganoza, 1966; Capecchi, 1967). This factor has been partially purified, shown probably to be protein (Capecchi, 1967), and fractionated into two independently acting components, one responding to the termination codons UAA and UAG and the other to UAA and UGA (Caskey *et al.*, 1968; Scolnick *et al.*, 1968).

The mechanism of the actual release reaction—the cleavage of the ester bond between nascent protein and tRNA—is still unknown. It seemed possible to us that this hydrolytic reaction might be carried out by a modification of the usual peptide-forming step of protein biosynthesis, which would result in the nascent peptide being transferred to water instead of to the amino group of an acceptor aminoacyl-tRNA, as in chain extension. This step is catalyzed by the enzyme peptidyl transferase, which is located in the ribosome; (Traut and Monro, 1964; Rychlik, 1966; Zamir *et al.*, 1966; Bretscher and Marcker, 1966); specifically, in the 50S subunit (Traut and Monro, 1964; Monro, 1967; Maden *et al.*, 1968).

In order to test this possibility we have employed a number of reagents and treatments that affect peptidyl transferase activity, and have compared, in parallel, their effect on peptide-bond formation with that on the release reaction. Peptide-bond formation was assayed with the puromycin reaction, in which 70S or isolated 50S ribosomes catalyze the synthesis of a peptide bond, with puromycin serving as the acceptor (Traut and Monro, 1964; Rychlik, 1966; Zamir *et al.*, 1966; Bretscher and Marcker, 1966; Monro and Marcker, 1967). Release activity was assayed according to Caskey *et al.* (1968). This convenient technique employs two separate trinucleotide codons instead of a single mRNA, but appears to represent the normal release reaction.

We have employed two main approaches. In the first we utilized the finding that the ability of the 50S ribosome to catalyze the synthesis of a peptide bond can be abolished and subsequently restored by appropriate mild treatments (Miskin *et al.*, 1968; Zamir *et al.*, 1969). In all cases the ability of such ribosomes to promote the release reaction ran parallel with their capacity for peptide-bond formation. In the second approach we tested a number of known inhibitors of the peptidyl transferase activity of the 50S ribosome. These inhibitors, which are effective at widely different concentrations, also inhibited the release reaction; and in each case the effective inhibitor concentration was virtually the same for both reactions.

These results indicate that the two reactions—peptide-bond synthesis and release—share a common component, and suggest that this component may be the peptidyl transferase of the 50S ribosomal subunit.

## Materials and Methods

*Antibiotics.* Lincomycin was kindly supplied as a gift by Dr. G. B. Whitfield, Upjohn, Kalamazoo, Mich., and Dr. J. B. Kirsch, Upjohn, Puurs, Belgium; amicetin by Dr. Whitfield; and sparsomycin by Dr. A. R. Stanley, Cancer Chemotherapy National Service Center, Bethesda, Md. Chloramphenicol, puromycin, and tetracycline were products of Parke Davis, Nutritional Biochemical Corp., and Teva, Israel, respectively.

Nitrous acid deaminated puromycin ("hydroxypuromycin," Vazquez and Monro, 1967) was prepared according to Hervé and Chapeville (1965), extracted with ether, and purified by filter paper electrophoresis for 30 min at 60 V/cm in 5% acetic

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acid brought to pH 3.5 with $NH_3$. The product was located with an ultraviolet lamp and eluted with water.

*N*-Acetylpuromycin was synthesized by acetylating 20 $\mu$-moles of puromycin·2HCl in 1.2 ml of 0.2 M triethanolamine (pH 7.5) with a fivefold excess of the acetyl ester of *N*-hydroxysuccinimide in 3 ml of ethyl acetate. The two-phase system was shaken 16 hr at room temperature and mixed with 0.5 ml of 1 M potassium phosphate (pH 8.3), and the ethyl acetate phase was removed. The aqueous phase was extracted with four 3-ml volumes of ethyl acetate. The product in the pooled extracts was adsorbed on a 0.8 × 28 cm column of silica gel in ethyl acetate, washed with 100 ml of ethyl acetate, and eluted with methanol–$H_2O$ (9:1). After evaporation the residue was taken up in 1-butanol–glacial acetic acid–$H_2O$ (5:2:3) and chromatographed on paper with the same solvent. The product was located with an ultraviolet lamp and eluted with the chromatography solvent.

*Ribosomes.* Ribosomes were prepared from 50-g batches of *Escherichia coli* MRE 600 (Cammack and Wade, 1965). They were sedimented three times in 120-ml volumes at 105,000*g* in the following media (with appropriate low-speed clarifications interspersed): (a) 4–5 hr in buffer 1 [60 mM $NH_4Cl$, 10 mM magnesium acetate, 10 mM Tris (pH 7.8), and 0.5 mM dithiothreitol]; (b) 4 hr in 0.5 M $NH_4Cl$, 0.1 mM magnesium acetate, and 10 mM Tris (pH 7.4), after having been stirred 14 hr in the same medium; (c) 2 hr in buffer 1, after an overnight dialysis against the same buffer. They were stored in buffer 1 at −180°. Such ribosomes showed a high but not absolute requirement for initiation factors; they would be expected to carry small amounts of other soluble factors. 30S and 50S subunits were prepared in buffer 2 [0.1 M $NH_4Cl$, 1 mM magnesium acetate, 10 mM Tris (pH 7.4), and 1 mM dithiothreitol] according to Atsmon *et al.* (1969).

*Inactivation and Reactivation of Ribosomes* (Miskin *et al.*, 1968; Zamir *et al.*, 1969). Ribosomal activities can be abolished and restored by suitable manipulation of temperature and the ionic composition of the medium (see Results for a fuller description). The present experiments are concerned with the ability of the 50S subunit, whether alone or as part of the 70S ribosome, to catalyze the formation of a peptide bond in the puromycin reaction (Traut and Monro, 1964; Rychlik, 1966; Zamir *et al.*, 1966; Bretscher and Marcker, 1966; Monro and Marcker, 1967). 70S ribosomes as prepared were active. They were inactivated by an overnight dialysis at 0–4° against two or three changes of a medium lacking $NH_4^+$ and $K^+$ (10 mM magnesium acetate, 10 mM Tris (pH 7.8), and 1 mM dithiothreitol); and were reactivated by being warmed at 30° for the times specified below in the same medium plus 0.1 M $NH_4Cl$. In some experiments reactivation was carried out after fMet-tRNA had been enzymatically bound to 70S ribosomes; in such cases the reactivation medium contained 0.1 M $NH_4Cl$, 30 mM $Mg^{2+}$, and 50 mM Tris (pH 7.2) plus initiation factors and other components of the binding mixture. 50S subunits as prepared were partially active. They were inactivated as described for the 70S ribosomes, except that the $Mg^{2+}$ concentration was 0.5 mM; and were reactivated at 30° in buffer 2 for the specified times. When chilled and stored in ice, reactivated ribosomes maintained a constant level of activity (R. Miskin, personal communication; Figure 5 below).

*Factors.* Release factor (Capecchi, 1967; Caskey *et al.*, 1968; Scolnick *et al.*, 1968) and initiation factors $F_1$ and $F_2$ (Revel and Gros, 1966; Stanley *et al.*, 1966; Eisenstadt and Brawerman, 1966) were isolated from the upper four-fifths of the $NH_4Cl$ ribosomal wash fluid. Although release factor was isolated from the supernatant fraction by the investigators cited, we found the ribosomal wash fluid to be a satisfactory source. The release factor preparations used were from an 0.5 M (a) and a 1.0 M wash (b). Both wash fluids gave the same results. The wash fluid was precipitated with $(NH_4)_2SO_4$ (0.4 g/ml), dissolved in and dialyzed against buffer 1, and centrifuged 20 min at 40,000*g*. The supernatant was diluted with buffer 1 and chromatographed on DEAE-Sephadex A-50 (Pharmacia) as shown in Figure 1. This procedure separated initiation factor $F_1$ from *N*-acyl-aminoacyl-tRNA hydrolase. A previously published procedure employing DEAE-cellulose did not separate the two proteins (Vogel *et al.*, 1968a). The present separation appears to be due to the gel filtration property of DEAE-Sephadex, since the hydrolase has been reported to be considerably larger than $F_1$ (Hershey *et al.*, 1969; Yot and Paulin, 1969). A large amount of ribonuclease activity, probably ribonuclease II (Spahr, 1964; Singer and Tolbert, 1964), emerges in the region of $F_2$ and release factor. This nuclease seriously interferes with experiments utilizing polynucleotide messengers. Although it did not affect our experiments, in which trinucleotide messengers were employed, we used fractions of $F_2$ and release factor that did not contain the nuclease. It should be noted, however, that our factor preparations were still relatively crude and were undoubtedly contaminated with other factors and enzymes not tested for.

*Assays and Preparations.* [$^{14}$C]fMet-tRNA was prepared by charging stripped *E. coli* W tRNA (Avital and Elson, 1969) at 3 mg/ml with [$^{14}$C]methionine (New England Nuclear, 218 or 233 mCi/mmole, 400 or 430 cpm per $\mu\mu$mole) in the presence of $N_5N_{10}$-methenyltetrahydrofolate and crude aminoacyl-tRNA synthetase (Rosenberg and Elson, 1969) for 15 min at 30° in 50 mM KCl, 50 mM Tris (pH 7.4), 10 mM magnesium acetate, and 2 mM ATP. AUG and UAG were synthesized from AU and UA (Waldhof, Mannheim) with polynucleotide phosphorylase and ribonuclease $T_1$ (Thach *et al.*, 1966). Ribosome concentration was estimated from the empirically found relationship: $(A_{260} - A_{290})/10$ = milligrams of ribosomes/milliliter, at pH 7–8. It was converted into molar concentration on the basis of assumed particle weights of $0.9 \times 10^6$, $1.8 \times 10^6$, and $2.7 \times 10^6$ for 30S, 50S, and 70S ribosomes. Protein was estimated from absorbance measurements at 280 and 260 m$\mu$ (Warburg and Christian, 1941), using a monograph by E. Adams (distributed by Calbiochem).

*Assays for Peptidyl Transferase and Release Activities.* Both assays were based on the release assay of Caskey *et al.* (1968). fMet-tRNA was bound to ribosomes enzymatically and the mixture was then diluted so as to increase the $Mg^{2+}$ concentration, dilute the initiation codon and factors, and, sometimes, introduce release factor. Binding was measured at this stage, after which puromycin was added to assay peptidyl transferase activity, or the termination codon UAG and, when not added before, release factor, to assay release activity. Except where stated otherwise, the details were as follows:

BINDING. The reaction mixture contained ribosomes, 8–9 mg/ml; initiation factors $F_1$ and $F_2$, 190 and 500 $\mu$g of protein per ml; [$^{14}$C]fMet-tRNA, 800–1200 $\mu\mu$moles/ml; AUG, $1.5 \times 10^{-5}$ M (0.5 $A_{260}$ unit/ml); magnesium acetate, 5 mM; $NH_4Cl$, 0.1 M; Tris (pH 7.2), 50 mM; and GTP, 1 mM. It was incubated at 0 or 30° as detailed in the figures and chilled in ice, and was diluted with a suitable volume of a solution such as to raise the $Mg^{2+}$

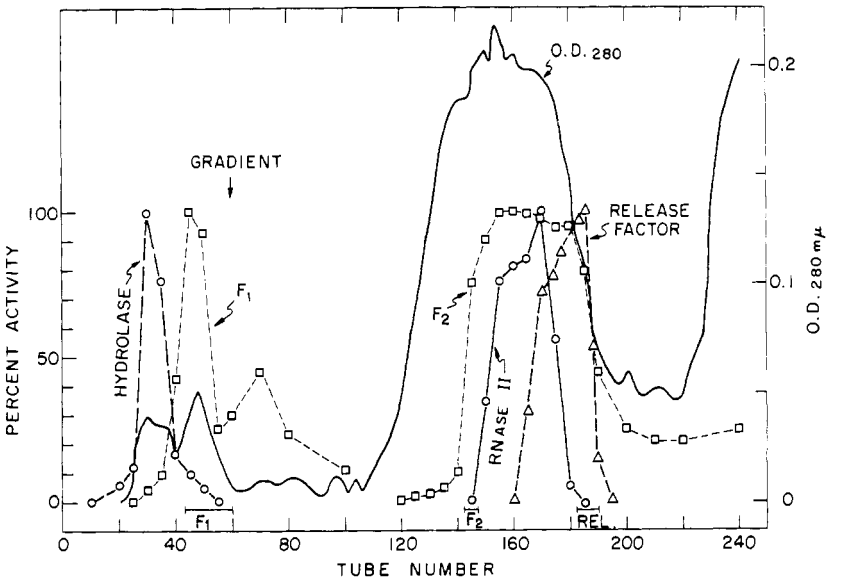


FIGURE 1: Chromatographic separation of release and initiation factors; 92.5 mg of protein from an 0.5 M $NH_4Cl$ ribosomal wash was diluted to 20 ml with buffer 1 and applied to a 3 × 40 cm column of DEAE-Sephadex A-50 which had been equilibrated with buffer 1. Elution was with 340 ml of buffer 1 followed by a 1.2-l. linear gradient of 0.06–0.65 M $NH_4Cl$ in buffer. 1. Fractions (6 ml) were collected at 0.5 ml/min. *N*-Acylaminoacyl-tRNA hydrolase and initiation factors $F_1$ and $F_2$ were assayed according to Vogel *et al.* (1968a); ribonuclease II according to Kivity-Vogel and Elson (1968); and release factor as described in the text, but for 30 min at 30°. Peak values are normalized to 100% and represent the following values (per milliliter): hydrolase, 360 units; $F_1$ and $F_2$, 48 and 112 μμmoles of fMet-tRNA bound; ribonuclease II, 8.2 μmoles of adenylic acid cleaved from polyadenylic acid in 10 min at 30°; release factor 28.7 μμmoles of fMet released. The entire eluate was tested for all activities, but only the active regions are shown in the figure. Thus, for example, the release factor was tested for hydrolase activity against *N*-carbobenzyloxyphenylalanyl-tRNA and found inactive, etc. The fractions used in experiments were taken as shown in the figure so as to avoid contamination of $F_1$ by hydrolase, and $F_2$ and release factor by ribonuclease II. $F_1$ was concentrated by vacuum dialysis against buffer 1. $F_2$ and release factor were precipitated with $(NH_4)_2SO_4$ (0.55 g/ml) and dissolved in and dialyzed against buffer 1. All factors were stored at −20 or −180°.

concentration to 30 mM, preserve the original concentrations of $NH_4Cl$ and Tris, and dilute the other components. Release factor was sometimes added here. Aliquots corresponding to 20 μl of the original binding mixture were taken to measure binding (Nirenberg and Leder, 1964). Identical aliquots were taken to assay peptidyl transferase and release activities, each at a final volume of 50 μl.

PEPTIDYL TRANSFERASE ASSAY. Puromycin was added (to 1 mM) and, if required, antibiotic. After incubation at 0° for the specified time, 1 ml of cold 1 M potassium phosphate (pH 7.2) (K. A. Marcker, personal communication) and 1.5 ml of ethyl acetate were added. After mixing and a brief centrifugation, 1 ml of the ethyl acetate phase was counted in 8 ml of Bray's fluid (Bray, 1960) in a Packard TriCarb liquid scintillation spectrometer. Values without puromycin (less than 0.1 μμmole) were subtracted. The reaction product, *N*-formylmethionylpuromycin, was completely extracted. The observed value was multiplied by 1.5 to correct for the fact that only 1 ml was counted.

RELEASE ASSAY. UAG was added (to 0.15 mM, 5 $A_{260}$ units/ml), together with release factor, if not added previously, and antibiotic, if required. The final concentration of release factor was 180 (preparation a) or 400 (preparation b) μg of protein per ml. After incubation at 0° for the indicated time, the reaction was stopped with 0.5 ml of cold 0.5 M sodium phosphate (pH 2.0) and extracted with ethyl acetate and counted as above. Background values obtained in the absence of UAG (about 1 μμmole) were subtracted. The omission of release factor or of both factor and UAG gave the same background value, which was constant at 0° for the duration of the experiments. At 30° the background increased with time, owing to chemical hydrolysis of fMet-tRNA. When necessary, a series of controls was run at different times (see, *e.g.*, Figure 6). The extraction of the reaction product, *N*-formylmethionine, was 65% effective, and the observed values were therefore multiplied by 2.3.

## Results

*Correlation of the Release Activity and Peptidyl Transferase Activity of Ribosomes.* The experiments were based on findings of Miskin *et al.* (1968) and Zamir *et al.* (1969), the relevant points of which are summarized here. These findings showed that 50S ribosomes lose their ability to catalyze peptide-bond synthesis when depleted of $NH_4^+$ and $K^+$ ions. Activity is restored if, in the presence of $Mg^{2+}$, either $NH_4^+$ or $K^+$ is replaced, but only if the ribosomes are also heated, suggesting a conformational change. Reactivation is extremely slow at 0°, appreciable at 20°, fast at 40°, and virtually instantaneous at 60°. By choosing appropriate conditions it is possible to bring inactive ribosomes to any desired degree of reactivation, and to maintain them at that level by chilling them to 0°. The inactivation–reactivation phenomenon is observed both when the 50S subunit is alone or when it forms part of a 70S ribosome. Under usual assay conditions (*e.g.*, incubation at 30 or 37° in a medium containing $Mg^{2+}$ and $K^+$ or $NH_4^+$), the phenomenon is obscured, since inactive ribosomes become reactivated under these conditions. In order to prevent this our assays were carried out at 0°.

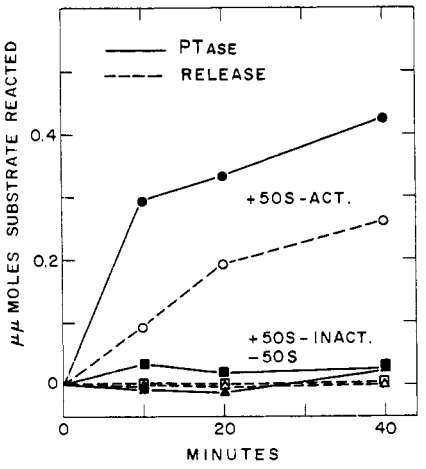


FIGURE 2: Peptidyl transferase and release activities of active and inactivated 50S ribosomes. fMet-tRNA was bound enzymatically to 30S ribosomes (30 μg, 33 μμmoles/assay tube) in 7 mM $Mg^{2+}$ at 30° for 15 min. Active or inactivated 50S ribosomes (67 μg, 37 μμmoles) were added at 0° and the mixtures were assayed. Release factor b, 8 μg of protein. Total binding of fMet-tRNA, 0.7 μμmole.

Similar phenomena are seen with the 30S subunit, whether alone or in the 70S ribosome, as detected by measuring the ability to bind aminoacyl-tRNA nonenzymatically in the presence of a suitable messenger (Nirenberg and Leder, 1964). In this case, however, the heat requirement for reactivation may be bypassed if the binding is carried out enzymatically. At 5 mM concentration of $Mg^{2+}$ in the presence of AUG and initiation factors, "inactive" ribosomes bind fMet-tRNA as effectively as active ribosomes at 0°. (The binding activities are different at other $Mg^{2+}$ concentrations.) Such enzymatic binding to "inactive" 70S ribosomes at 0° does not affect the peptidyl transferase activity of the 50S subunit, which remains inactive. We have exploited this finding in the experiments described below.

The experimental procedure employed here was based on the termination assay of Caskey *et al.* (1968). fMet-tRNA was bound enzymatically at 0° to ribosomes of different states of peptidyl transferase activity, and the mixture was adjusted to 30 mM $Mg^{2+}$ in order to stabilize the binding complex and dilute the initiation codon and factors. It was then assayed either for peptidyl transferase activity by adding puromycin (the product is fMet-puromycin), or for release activity by adding the termination codon UAG and release factor (the product is fMet). Experimental details are given in Materials and Methods and the figures.

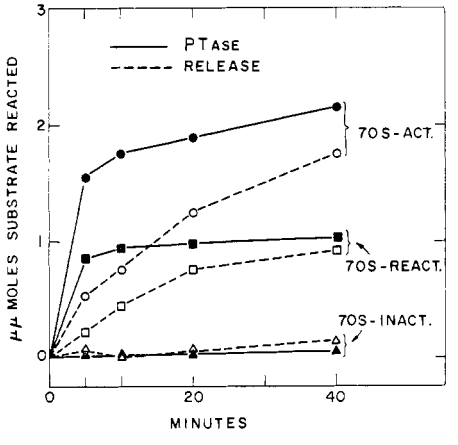


FIGURE 3: Peptidyl transferase and release activities of active, inactivated, and reactivated 70S ribosomes. fMet-tRNA was bound enzymatically to active (A), inactivated (I), or 10-min reactivated (R) 70S ribosomes (175 μg, 65 μμmoles/assay tube) at 0° for 15 min and the mixtures were assayed. Release factor b, 20 μg of protein. Total binding of fMet-tRNA (μμmoles): A, 3.3; I, 10.1; R, 2.1.

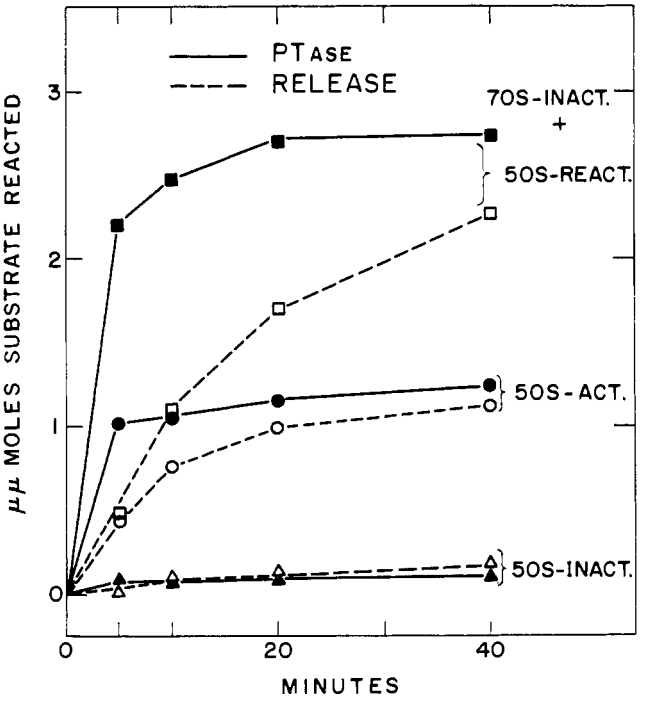


FIGURE 4: Peptidyl transferase and release activities of inactivated 70S ribosomes mixed with active, inactivated, or reactivated 50S ribosomes. Inactive 70S ribosomes (90 μg, 33 μμmoles/assay tube) were mixed with 50 μg (28 μμmoles) of active (A), inactivated (I), or 10-min reactivated (R) 50S ribosomes. fMet-tRNA was bound at 0° for 15 min and the mixtures were assayed. Release factor b, 20 μg of protein. Total binding of fMet-tRNA (μμmoles): A, 7.4; I, 6.1; R, 7.8.

*Experiments with Isolated Subunits.* In early experiments, fMet-tRNA was first bound to isolated 30S subunits at 30°, after which either active or inactivated 50S subunits were added at 0° and the mixtures were assayed. As shown in Figure 2, 50S subunits that exhibited peptidyl transferase activity were also capable of promoting the release reaction, while their inactivation abolished both activities. The accompanying 30S subunits were active in all cases, since the binding reaction had been carried out at 30°. The results show that active 50S subunits are required for release.

*Experiments with 70S Ribosomes.* Although the results obtained in the above experiments were clear, the binding of fMet-tRNA to isolated 30S subunits was poor and the assay results were based on relatively low levels of radioactivity (see Figure 2). Since 70S ribosomes bound fMet-tRNA much more effectively under the conditions employed, they were used in succeeding experiments.

Figure 3 shows an experiment in which fMet-tRNA was

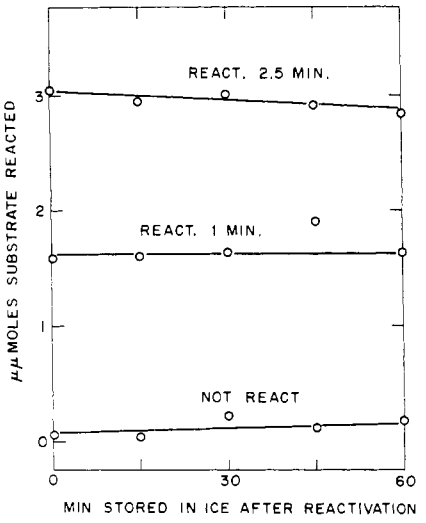


FIGURE 5: Stability of the peptidyl transferase activity of partially reactivated ribosomes at 0°. fMet-tRNA was enzymatically bound to inactive 70S ribosomes (175 μg, 65 μμmoles/assay tube) for 30 min at 0°. Portions of the complex were reactivated at 30° for 1 or 2.5 min and immediately placed in ice. Thereafter samples were taken for peptidyl transferase assay after 0, 15, 30, 45, and 60 min. Each assay was run for 10 min at 0°, by which time the reaction is complete. Total binding of fMet-tRNA(μμmoles): 0 min, 7.2; 1 min, 7.3; 2.5 min, 7.7.

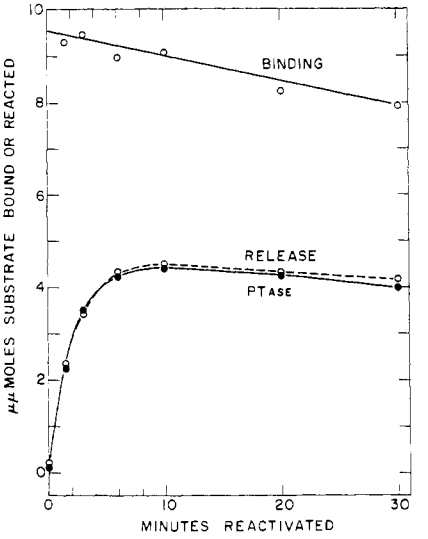


FIGURE 6: Correlation of the restoration of peptidyl transferase and release activities by heat reactivation of ribosomes. fMet-tRNA was enzymatically bound to inactive 70S ribosomes (175 μg, 65 μμmoles/assay tube) at 0° for 30 min. A portion of the complex was left in ice. Other portions were reactivated at 30° for 1.5, 3, 6, 10, 20, and 30 min and returned to ice. All were then assayed for 60 min in order to allow both reactions to approach completion, so as to measure extent of reaction. Since the extent of chemical hydrolysis of fMet-tRNA increased with increasing reactivation time (presumably accounting for the steady decrease in binding), controls lacking UAG were run for each time point of the release curve, and subtracted. Release factor b, 20 μg of protein.

bound enzymatically at 0° to active, inactivated, or reactivated 70S ribosomes. As before, release activity ran parallel to peptidyl transferase activity, being abolished by the mild inactivation treatment and restored to the same degree by the reactivation treatment. The two reactions proceed at different rates. The release reaction is slower, presumably because it is the more complex reaction and involves at least two steps not required by the puromycin reaction: the binding of a termination codon and of release factor. Nevertheless, both reactions tend toward the same final plateau value, indicating that equal amounts of substrate react in both cases.

*Experiments with Inactive 70S Ribosomes and Added 50S Subunits.* The above experiments were conducted in such a way that wherever release activity was observed, not only the 50S subunit but also the 30S subunit was active or had been brought to an active state with heat. While they showed that active 50S subunits are required for release, the experiments did not exclude the possibility that the 30S unit must also be active. By resorting to enzymatic binding in the cold, it was possible to test inactive 30S subunits that had not undergone heat activation. As mentioned above, the enzymatic binding of fMet-tRNA to isolated 30S subunits was relatively poor at 0°; we therefore performed the experiment as described in Figure 4 so as to take advantage of the high binding capacity of 70S ribosomes.

Inactive 70S ribosomes were mixed in the cold with 50S subunits that were either active, inactivated, or reactivated, and enzymatic binding was then carried out in the cold. The results show that mixed ribosomes were obtained, made up of inactive 30S and active 50S where this was possible. It is not clear whether this occurred through the dissociation and reassociation of subunits before binding, or the exchange of 50S subunits after binding. In any event, the release and peptidyl transferase activities were again parallel in all cases, and were present only when the added 50S subunits were active or reactivated. These results show that an active 50S subunit is obligatory for the release reaction, but that the 30S subunit is functional in this reaction even when it has not undergone heat reactivation. They leave open the question of whether interaction with initiation factors at 0° causes changes in the 30S subunit comparable with those produced by heat.

*Partial Reactivation.* As mentioned above, it is possible to reactivate ribosomes to varying degrees. We therefore tested whether partial reactivation of inactivated ribosomes would restore both activities to the same degree. fMet-tRNA was enzymatically bound to inactive 70S ribosomes at 0°, the $Mg^{2+}$ concentration was raised to 30 mM, and the complex was warmed in a reactivation medium at 30°. Samples were placed in ice after various times and subsequently assayed. A control experiment showed that such partially reactivated complexes, when kept in ice, maintain an unchanging level of peptidyl transferase activity for at least an hour (Figure 5).

In the experiment itself (Figure 6), portions of the originally inactive complex were reactivated at 30° for different periods of time and returned to ice. They were then assayed for both activities in the usual way, except that the assay incubation was extended to 60 min in order to allow both reactions to reach completion. What was measured, therefore, was not the rate of the reactions but their extent, *i.e.*, the fraction of the ribosomes that had been reactivated. It is seen that both activities were restored to exactly the same degree over the entire reactivation range.

In this experiment 50% of the bound substrate underwent reaction. For reasons not yet understood, this fraction has varied from one experiment to another, being sometimes less

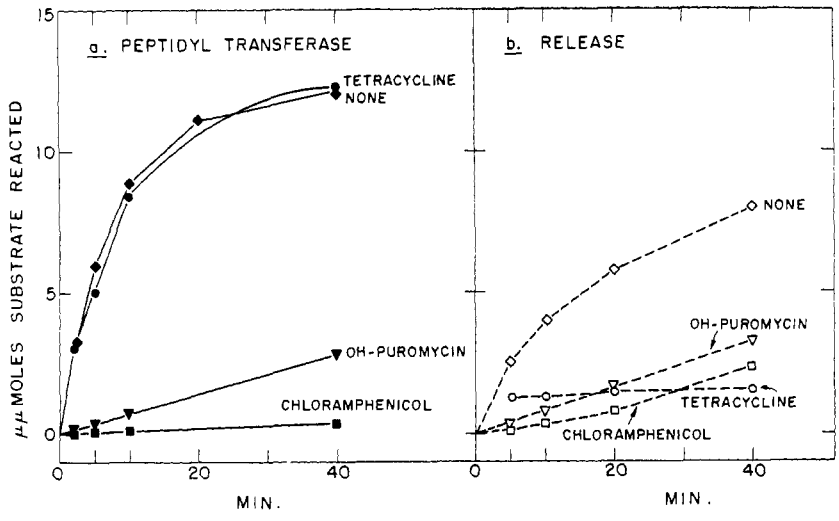


FIGURE 7: Effect of inhibitors on peptidyl transferase and release activities. fMet-tRNA was bound to active 70S ribosomes (175 μg, 65 μμmoles/assay tube) at 30° for 15 min. The mixtures were put in ice and assayed. Release factor *b*, 20 μg of protein/tube; puromycin, $2.5 \times 10^{-5}$ M; inhibitors, $3 \times 10^{-4}$ M. None, no inhibitor. Total binding of fMet-tRNA (μμmoles): peptidyl transferase assay, 13.3; release assay, 11.8.

then 50% and sometimes more, occasional y reaching 100%. Where both release and peptidyl transferase activities were assayed in the same experiment, the same fraction of bound substrate reacted in both cases.

During these experiments it was observed (R. Miskin and Z. Vogel, unpublished) that the rate of heat reactivation of peptidyl transferase activity is considerably higher when inactive 70S ribosomes carry bound f-Met-tRNA (as in Figure 6) than when they do not. Whatever the nature of this effect may be, the experiment of Figure 6 shows it to affect the release and peptidyl transferase activities equally.

*Comparison of the Effect of Inhibitors on the Peptidyl Transferase and Release Activities of Ribosomes.* As a second method of examining the relationship between the release and peptidyl transferase activities, we turned to the use of antibiotics known to interfere with peptide-bond synthesis, a number of which interact with the 50S ribosomal subunit and inhibit peptidyl transferase (see Weisblum and Davies, 1968, for a comprehensive review and references). We have examined several of these inhibitors in order to compare their effects on the peptidyl transferase and release activities of active ribosomes. The experimental procedure employed was that used in the experiments described above, except that inhibitor was added to the reaction mixtures.

Figure 7 shows the effects of three antibiotics on the two ribosomal activities. Chloramphenicol is known to bind to the 50S subunit and inhibit peptidyl transferase activity (Vazquez, 1964; Wolfe and Hahn, 1965; Weisblum and Davies, 1968). It has also been shown to inhibit the release reaction (Scolnick *et al.*, 1968). Tetracycline, a 30S inhibitor, does not affect peptidyl transferase activity (Weisblum and Davies, 1968) but does inhibit release, presumably by interfering with a step involving the 30S subunit (Scolnick *et al.*, 1968). Our results confirm these observations. Hydroxypuromycin is believed to affect the 50S subunit (Vazquez and Monro, 1967) and may be assumed to interact with peptidyl transferase, since it is a close analog of a substrate of the enzyme, puromycin. Figure 7 shows it to inhibit both the peptidyl transferase and release activities.

Figure 8 shows the inhibitory effect of six antibiotics as a function of antibiotic concentration. Five of them—hydroxypuromycin, chloramphenicol, sparsomycin, lincomycin, and amicetin—are known to interact with the 50S subunit and inhibit peptidyl transferase activity (Monro and Vazquez, 1967; Weisblum and Davies, 1968). Our results show that *N*-acetylpuromycin also does. It can be assumed to interact directly with peptidyl transferase, but is a poorer inhibitor than hydroxypuromycin, presumably because it is a less close analog of puromycin. It has been suggested that sparsomycin may also interact directly with peptidyl transferase (Monro *et al.*, 1969; Goldberg and Mitsugi, 1967).

Each of the six antibiotics inhibited both activities. Furthermore, although the effective concentrations of different antibiotics were widely different, each individual antibiotic inhibited the two reactions over the same concentration range and to about the same degree at each concentration tested. This suggests, again, that the release and peptidyl transferase activities share a common component that is affected by these inhibitors.

## Discussion

Our results show that the ability of an *in vitro* ribosomal system to release nascent peptide in the presence of a termination codon and release factor runs parallel with the peptidyl transferase activity of the 50S ribosomal subunit. The evidence is of two kinds: (a) Inactivation of the 50S subunit eliminates both activities; reactivation restores both activities; and partial reactivation restores both activities partially and to the same degree. (b) Antibiotics known to inhibit the peptidyl transferase activity of the 50S subunit also inhibit the release activity, and with the same dependence on antibiotic concentration.

There may well be a direct connection between these two phenomena, the state of activity of the 50S ribosome and the effect of antibiotics on it. In experiments to be published elsewhere we have found that chloramphenicol binds only to active 50S subunits, as determined directly with radioactive chloramphenicol. The same appears to be true of sparsomycin, since only active 50S subunits form the hyperstable complex with the terminal fragment of *N*-acetylleucyl-tRNA, the formation of which is believed to depend on the binding of sparsomycin (Monro *et al.*, 1969). In both cases, inactivation of the 50S subunit abolishes the binding, and reactivation re-

stores it. There appears to be, therefore, a connection between the conformational changes in the 50S subunit induced by heat and monovalent cations, the binding of antibiotics, peptidyl transferase activity, and release activity. They may all have to do with the same region of the 50S ribosome.

Our results not only implicate the 50S ribosomal subunit as an active participant in the release reaction (see also Scolnick *et al.*, 1969), but also suggest that the 50S ribosomal enzyme peptidyl transferase may be directly involved. However, the dual requirement for active 50S subunits and release factor may still be interpreted in more than one way. For example, the release factor might itself cleave the bond between nascent peptide and tRNA. The factor has been shown to be unable to do this when free in solution (see, *e.g.*, Figure 1), but it might be able to catalyze the reaction when the substrate is bound to a ribosome; and the factor itself might be able to bind to the ribosome only when the 50S subunit is in an active conformation. Alternatively, peptidyl transferase might cleave the substrate, with the release factor acting to change the specificity of the peptidyl transferase reaction, so that nascent peptide is now transferred to water instead of to a molecule of aminoacyl-tRNA. In this event, the release factor might interact directly with peptidyl transferase, in which case the factor could be looked on as part of a multimeric release enzyme; or it might interact with another region of the ribosome.

It was previously suggested that the nonribosomal enzyme *N*-acylaminoacyl-tRNA hydrolase may catalyze the release reaction (Cuzin *et al.*, 1967; Vogel *et al.*, 1968a; de Groot *et* al., 1968; Kössel and RajBhandary, 1968). This enzyme hydrolyzes free but not ribosome-bound peptidyl-tRNA (de Groot *et al.*, 1968) and does not require ribosomes, release factor or termination codon for its activity. Consequently, it would be required that peptidyl-tRNA be detached from the ribosome, perhaps by release factor, before being cleaved by the hydrolase. This now seems unlikely in view of the results of Caskey *et al.* (1968), who showed that fMet-tRNA is cleaved during the release reaction, while it is known that free fMet-tRNA is a very poor substrate for the hydrolase (Vogel *et al.*, 1968a,b; Kössel and RajBhandary, 1968; Lapidot *et al.*, 1969). Furthermore, preliminary experiments in this laboratory indicate that the rate of release is not affected by extreme variations in the hydrolase concentration (Z. Vogel, unpublished). Thus, there is no evidence that connects the hydrolase with the release reaction.

In conclusion, we have shown that the release reaction requires active 50S ribosomal subunits in addition to the previously known requirements for release factor and a termination codon. Our results suggest (but do not prove) that the peptide-bond-forming enzyme, peptidyl transferase, may also catalyze the hydrolytic release reaction.

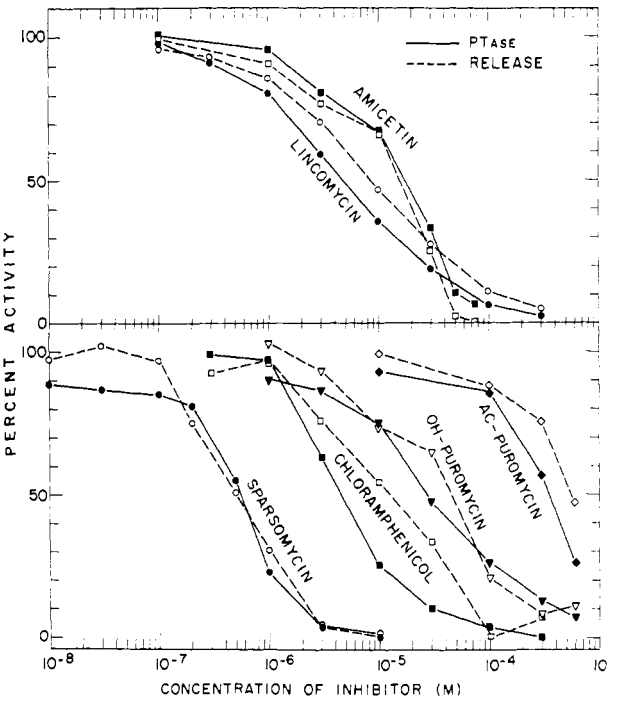


FIGURE 8: Effect of inhibitor concentration on peptidyl transferase and release activities. fMet-tRNA was bound to active 70S ribosomes (175 $\mu$g, 65 $\mu\mu$moles/assay tube) at 30° for 15 min. The mixtures were put in ice and assayed in the presence of different inhibitor concentrations. The results are taken from two separate experiments: expt 1, amicetin, hydroxypuromycin, *N*-acetylpuromycin, chloramphenicol; expt 2, lincomycin, sparsomycin. In each experiment the two activities were determined with separate binding mixtures, one immediately after the other. *Peptidyl transferase assay:* $1 \times 10^{-4}$ M puromycin, 1 min. *Release assay:* release factor b, 20 $\mu$g of protein/tube (expt 1) or factor a, 9 $\mu$g/tube (expt 2); 20 min. The assay times were chosen to limit the reactions to about 30% or less of the bound substrate, so as to measure rate of reaction. The reaction was slower than that shown in earlier figures, since the release factor employed had lost about half of its original activity after being stored for half a year. The solution of sparsomycin employed was an old one which had lost much activity; fresh solutions were active at much lower concentrations, but in all cases were equally effective against both reactions; $\mu\mu$moles of fMet-tRNA bound and (in parentheses) $\mu\mu$moles that reacted in the absence of inhibitor (100% activity): peptidyl transferase: expt 1, 6.86 (1.77); expt 2, 6.02 (1.67); release: expt 1, 6.49 (1.94); expt 2, 6.91 (2.17).

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