

## Mechanism of Puromycin Action: Fate of Ribosomes after Release of Nascent Protein Chains from Polysomes

(subunit exchange/spermidine/protein synthesis/ribosome cycle)

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Communicated by Luis F. Leloir, August 15, 1973

**ABSTRACT** The exchange of ribosomal subunits during the release of growing polypeptide chains by puromycin has been investigated in a bacterial cell-free system engaged in protein synthesis. The addition of spermidine, used as a stabilizing agent of 70S monomers, caused a strong inhibition of the subunit exchange. This result led us to conclude that upon premature release of unfinished protein chains by the antibiotic, the ribosomes fall off mRNA as 70S particles. This behavior is different from that occurring during physiological termination of translation, where the ribosomes detach in a dissociated form. Some implications of the postulated mechanism are also discussed.

Puromycin is a well-known inhibitor of protein synthesis *in vivo* as well as in cell-free systems. Its structure, analogous to aminoacyl-tRNA, leads to the premature release of unfinished polypeptide chains as polypeptidyl-puromycin derivatives (1-4).

However, the fate of ribosomes upon release of growing protein chains by puromycin is still not well understood. Schlessinger *et al.* (5) reported that ribosomes engaged in polyphenylalanine synthesis were dissociated into subunits after treatment with puromycin, because the free 30S-50S couples were unstable. More recently Kohler *et al.* (6) could not confirm these results; they found that 70S particles bearing polyphenylalanyl-tRNA remained intact when peptide chains were liberated by the antibiotic.

Both experiments lead to opposite conclusions, but neither of them can be used as a good model of the puromycin reaction at the polysomal level, which constitutes a more physiological system.

On the other hand, the addition of puromycin to growing bacterial cultures causes the breakdown of polyribosomes with a concomitant accumulation of 70S monomers (6-8). These particles behave as run-off ribosomes with respect to their stability at different  $Mg^{++}$  ion concentrations (9). This fact is consistent with the idea that ribosomal particles fall off mRNA by the action of the antibiotic.

The subunit exchange observed in a cell-free system for protein synthesis is accelerated by the presence of puromycin (10). This phenomenon has been interpreted as depending on the eventual capacity of ribosomes to reinitiate translation or at least to reassemble around a mRNA molecule.

In our studies on termination of protein synthesis we have measured the exchange of subunits in the presence and absence of spermidine. This comparison led us to the conclusion that upon physiological termination, when completed polypeptides are released, the ribosomal particles detach from mRNA as

subunits, which can then reassociate to form 70S monomers (11).

Peptidyl-puromycin formation is commonly used as a model reaction in the studies on polypeptide elongation and several other aspects of the ribosome cycle; it is worthwhile then to elucidate the fate of ribosomal particles when puromycin is added to a complete system engaged in protein synthesis. For this purpose, we have also used experiments of subunit exchange with and without spermidine. This is a useful tool to investigate whether or not the ribosomes are liberated from polysomes in a dissociated form after premature release of unfinished proteins.

### MATERIALS AND METHODS

Puromycin·(HCl)<sub>2</sub> was obtained from Sigma, tetracycline·HCl from Lepetit (Argentina), sparsomycin from Upjohn Co., fusidic acid from Leo Pharmaceutical Products (Denmark), and spermidine phosphate from Mann.

<sup>14</sup>C-Labeled amino acids (reconstituted protein hydrolysate) were purchased from Schwarz BioResearch, [<sup>14</sup>C]leucine (300 Ci/mol) from New England Nuclear Corp., [<sup>14</sup>C]uracil (50 Ci/mol) from the Commissariat à l'Energie Atomique (France), and [<sup>32</sup>P]orthophosphoric acid (carrier free) from Comisión Nacional de Energía Atómica (Argentina).

The preparation and purification of polyribosomes and radioactive 30S subunits from *Escherichia coli* D<sub>10</sub> have been reported previously (11, 12). The assays for polypeptide synthesis and subunit exchange have also been described in detail (11). The standard reaction mixtures contained, in a total volume of 0.125 ml, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer of pH 7.4, 8 mM magnesium acetate, 64 mM KCl, 1.4 mM ATP, 0.3 mM GTP, 8 mM *P*-enolpyruvate, 16 mM 2-mercaptoethanol, a complete amino-acid mixture (0.1 mM each), S<sub>100</sub> supernatant fluid (0.1-0.4 mg of protein) and 1.0-3.0 A<sub>260</sub> units of purified polyribosomes, with the addition of [<sup>14</sup>C]amino acids or radioactive 30S particles in order to measure protein synthesis or subunit exchange, respectively. Unless otherwise indicated, the exchange was expressed as the percentage of the total radioactivity initially present in the 30S subunits that sediments with the 70S monomer after the completion of the reaction and subsequent analysis by sucrose gradient centrifugation.

*Preparation of Polyribosomes Labeled with Nascent [<sup>14</sup>C]-Peptides or [<sup>32</sup>P]mRNA.* *E. coli* D<sub>10</sub> cultures in the early exponential phase were subjected to a 90-sec pulse with [<sup>14</sup>C]-

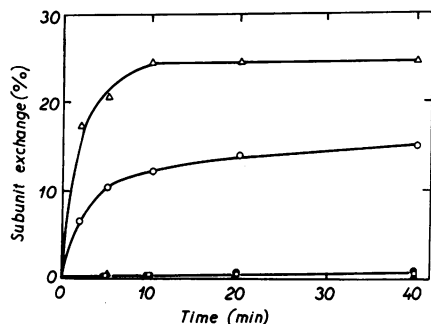


Fig. 1. Effect of several antibiotics on the kinetics of subunit exchange during protein synthesis. The cell-free system from *E. coli* D<sub>10</sub> and the assay conditions for subunit exchange measurements were described elsewhere (11, 12). Results are expressed as indicated in *Methods*. Complete system without additions (O—O); complete system in the presence of 1.2 mM puromycin (Δ—Δ), 0.36 mM sparsomycin (□—□), 1.2 mM tetracycline (●—●), or 1 mM fusidic acid (▲—▲).

leucine (25  $\mu$ Ci/liter) or [<sup>32</sup>P]orthophosphate (1 mCi/liter of medium) and the growth was immediately stopped by very fast cooling. Other conditions employed for harvesting, lysis, and polysome purification have already been reported (11).

## RESULTS

*Exchange of Subunits in the Presence of Puromycin.* The cell-free system used in all the experiments to be described in this paper contained purified polyribosomes and all other components required for elongation, completion, and partial release of the synthesized polypeptide chains. However, such a system was unable to reinitiate protein synthesis, as has been shown before (12). This conclusion is based on the following results: (1) none of the radioactivity from labeled subunits appeared in the polysomal region of sucrose gradient profiles after long or short incubations; (2) polypeptide synthesis and subunit exchange did not decrease when the supernatant fluid used in the reaction mixtures was prepared from bacteria subjected to a treatment with trimethoprim in order to deplete the fMet-tRNA pool. (Under these conditions the process of initiation of polypeptide synthesis with natural mRNA is completely blocked.)

We have studied the effect of several antibiotics on the subunit exchange during protein synthesis (Fig. 1). Tetracycline, sparsomycin, and fusidic acid inhibit to the same extent both the exchange of ribosomal subparticles and the amino acid incorporation into polypeptides (10, 13). In contrast, puromycin, which is also a strong inhibitor of protein synthesis, markedly increased the subunit exchange. Moreover, there was a good correlation between the inhibition of polypeptide synthesis and the increase of exchange at different concentrations of puromycin (Fig. 2).

*Ribosome Detachment from mRNA upon Release of Nascent Peptides by Puromycin.* Polyribosomes containing labeled mRNA were incubated in a complete mixture for elongation and termination of polypeptides. Subsequently, the samples were analyzed by sucrose gradient centrifugation under conditions that allow for a good resolution of monomers, ribosomal subunits, and lighter fractions, while the peaks corresponding to trisomes and heavier aggregates have already reached the bottom of the tube.

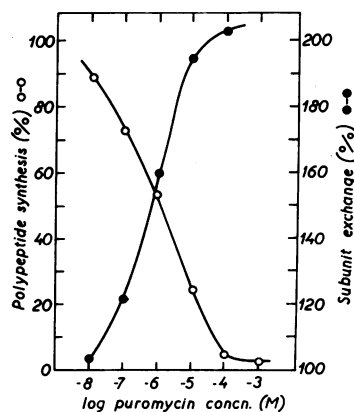


Fig. 2. Effect of puromycin concentration on polypeptide synthesis and subunit exchange. Assays for polypeptide synthesis and exchange were carried out with <sup>14</sup>C-labeled amino acids and <sup>32</sup>P-labeled 30S ribosomal subunits, respectively, in the presence of the indicated concentrations of antibiotic. All mixtures were supplemented with 0.3 mg of stripped tRNA from *E. coli* W. The values arbitrarily taken as 100% correspond to the experiments performed in the absence of puromycin (6800 cpm of amino-acid incorporation and 14.2% of subunit exchange, respectively).

Fig. 3 shows that the release of mRNA (or its fragments) into the supernatant fluid and the accumulation of 70S particles were greatly increased by the addition of puromycin. At the same time polyribosomes were broken down more rapidly.

In the experiments which are described in the next section we have measured the subunit exchange in the presence and absence of spermidine. Therefore, several controls were carried out to demonstrate that the liberations of mRNA and unfinished polypeptides by puromycin, which were of the same order, did not change when the polyamine was added to the assays (Fig. 3C and D, and Table 1). These controls were necessary to be sure that spermidine did not stabilize any complex between ribosomal particles and mRNA or growing protein chains.

*Spermidine Inhibits the Exchange Induced by Puromycin.* The exchange of ribosomal subunits in a complete system for protein synthesis has been measured in the presence of <sup>32</sup>P-labeled 30S subunits and puromycin, with and without the addition of spermidine. If, upon treatment with the antibiotic, ribosomes were dissociated in order to detach from mRNA, the polyamine should not inhibit the subunit exchange, unless the resultant subparticles were unable to equilibrate with the pool of subunits. [This possibility was ruled out by the experiments carried out in the absence of puromycin (11).] On the contrary, if detachment occurs as 70S particles, spermidine would stabilize the monomers and the exchange would be blocked. The results depicted in Fig. 4A are in full agreement with the second alternative. Fig. 4B shows that "post termination" exchange, measured by adding radioactive subunits after 10 min of incubation in the presence of puromycin, is also markedly reduced by spermidine, whereas it occurs very rapidly in the absence of polyamine.

## DISCUSSION

The increase of subunit exchange induced by puromycin has been interpreted in several ways by Kaempfer and Meselson (10). These authors have proposed two possibilities: (a) The

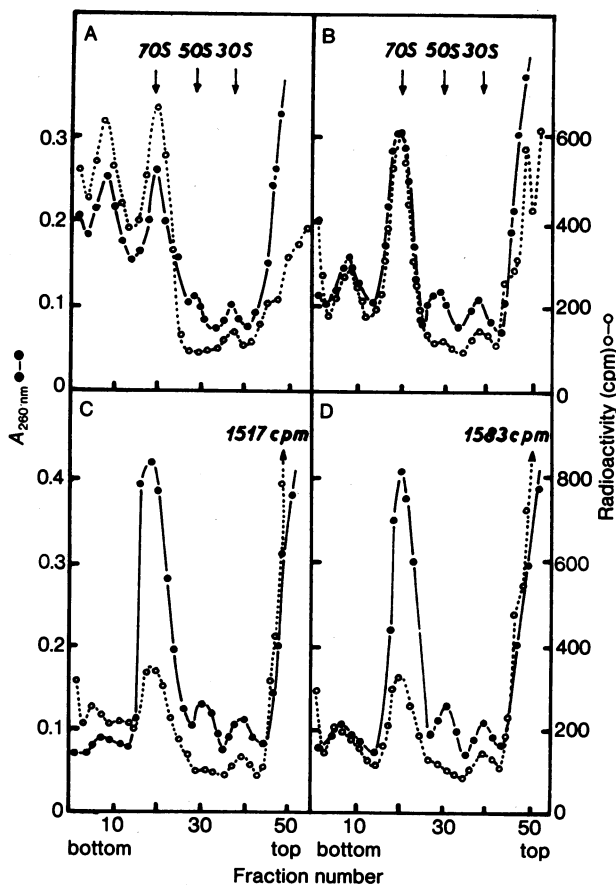


FIG. 3. Detachment of ribosomal particles from mRNA upon physiological termination or premature release of peptides by puromycin. Standard mixtures for polypeptide synthesis containing 2.7  $A_{260}$  units of polysomes with  $^{32}\text{P}$ -labeled mRNA were incubated as indicated in each case, chilled, and immediately layered on 4.6 ml of 5–20% linear sucrose gradients. After centrifugation during 90 min at 40,000 rpm in a SW 65 rotor, gradients were fractionated from the bottom of the tubes, and the  $A_{260}$ , as well as the radioactivity of each fraction, was measured. (A) Complete mixture at 0°; (B) after 10-min incubation at 37°; (C) the same as in B but in the presence of 1.2 mM puromycin; (D) the same as in B but in the presence of 1.2 mM puromycin plus 1 mM spermidine.

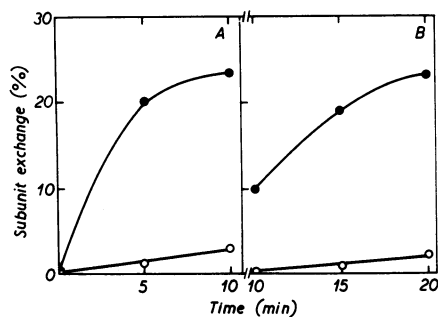


FIG. 4. Effects of spermidine on the exchange of subunits during or after premature termination induced by puromycin. Reactions were performed as described previously (12) with the addition of 1.2 mM puromycin, in the absence (●—●) or in the presence (○—○) of 1 mM spermidine. Radioactive 30S subunits were added either from the beginning of the reaction (A) or after 10 min of incubation of 37° (B).

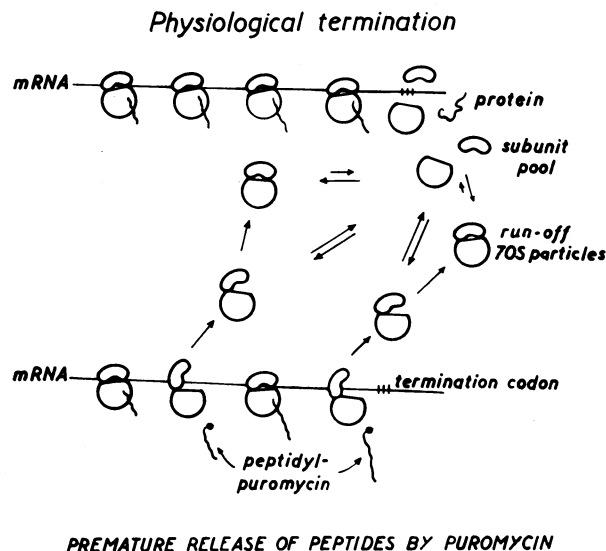


FIG. 5. Proposed mechanism of detachment of ribosomes from polysomes during physiological termination of polypeptide chains or puromycin action.

drug would not only release peptide chains, but also those 50S subunits involved in the process, leaving their 30S partners associated with mRNA. Then the smaller subunits still bound to mRNA could recombine with other 50S subunits to synthesize fragments of protein chains. (b) Puromycin would release both subunits, which could reassociate at initiator codons after equilibration with the pool of subunits.

Both these possibilities are based on the assumption that subunit exchange takes place only at the level of mRNA complexes and requires protein synthesis. However, it has been shown that this hypothesis is not correct, because run-off 70S monomers are also able to undergo subunit exchange through their equilibrium with the pool of free subunits (11, 14).

In our experiments we have used an *in vitro* system which can neither initiate polypeptide synthesis nor bind 30S subunits to mRNA. Therefore, the exchange of ribosomes with added radioactive 30S subunits can occur only after detachment of ribosomal particles from the mRNA. Fig. 3 and Table

TABLE 1. Peptide release and ribosome detachment from mRNA produced by termination of translation or puromycin action

Additions	Release of peptides (%)	Release of mRNA (%)
None	32	38
Puromycin	79	82
Puromycin and spermidine	81	84

The experiments were performed with the complete mixtures for polypeptide synthesis containing 2.7  $A_{260}$  units of polysomes bearing  $^{14}\text{C}$ -labeled growing polypeptides (5130 cpm) or  $^{32}\text{P}$ -labeled mRNA (30,000 cpm). Where indicated, 1.2 mM puromycin and 1 mM spermidine were added. After incubation for 20 min at 37°, samples were submitted to sucrose gradient centrifugation and the radioactivity liberated into the supernatant fluid (fractions lighter than the 30S peak) was measured. These values are expressed as percentages of the total radioactivity in each case.

I demonstrate that the release of ribosomes from polysomes actually takes place, and Figs. 1 and 4B show that these particles are rapidly engaged in the exchange with the pool of subunits.

Although our system may not reproduce exactly the physiological conditions, it is interesting to point out that  $Mg^{++}$  and polyamine concentrations are probably in the same range as the *in vivo* levels.

The use of spermidine in the studies on subunit exchange made it possible to conclude that, whereas during natural termination of protein synthesis the ribosomes detach from mRNA in a dissociated form, they are liberated as 70S monomers upon the release of incomplete peptide chains by puromycin (Fig. 4).

Perhaps the physiological termination of translation involves a more drastic conformational change (leading to dissociation of ribosomes) than the one required for the interaction between puromycin and nascent polypeptides. In this case we could envisage both subunits opening to fall off mRNA, while still maintaining a linkage between them, as outlined in Fig. 5.

Although fusidic acid does not inhibit polypeptidyl-puromycin formation (15), it abolished the subunit exchange during natural termination (Fig. 1), as well as the increased exchange due to the presence of puromycin. Furthermore, inhibitors of translocation also block polysome breakdown in the presence of puromycin (16).

All these facts strongly suggest that ribosomes move along the messenger beyond the point of peptide release by the drug, and one, a few, or many translocations are needed before the detachment of ribosomal particles from polysomes occurs.

#### NOTE ADDED IN PROOF

After this paper was sent for publication, the work of A. R. Subramanian and B. D. Davis on release of 70S ribosomes from polysomes [*J. Mol. Biol.* (1973) **74**, 45-56] reached our hands. Although we have used a more physiological system,

our results agree with the conclusions of Subramanian and Davis related to the release by puromycin. This is not the case for natural termination; we suggest a different mechanism for this process on the basis of previous results from our laboratory (11). The conditions used by Subramanian and Davis (elevated Mg concentration or lower temperature) may alter the normal runoff.

We are grateful to Drs. L. F. Leloir, G. Favelukes, B. Friedlander, and all members of the Instituto de Investigaciones Bioquímicas for valuable discussions. This work was supported in part by grants from the University of Buenos Aires and the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina). I.D.A. is a career investigator of the latter institution, and M.E.A. is a former fellow of the Instituto Nacional de Farmacología.

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